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(54) Title: PROTEIN

(57) Abstract: The invention also encompasses the use of a lipolytic enzyme obtainable from one of the following genera: *Streptomyces*, *Corynebacterium* and *Thermobifida* in various methods and uses, wherein said lipolytic enzyme is capable of hydrolysing a glycolipid or a phospholipid or transferring an acyl group from a glycolipid or phospholipids to a acyl acceptor. Preferably, the lipolytic enzyme for use in these methods and uses comprises an amino acid sequence as shown in any one of SEQ. ID. No.s 4, 5, 7, 8, 12, 14 or 16 or an amino acid sequence having at least 70% identity therewith or comprises a nucleotide sequence shown as SEQ ID No. 3, 6, 9, 13, 15 or 17 or a nucleotide sequence which has at least 70% identity therewith. The present invention also relates to a lipolytic enzyme capable of hydrolysing at least a galactolipid or capable of transferring an acyl group from a galactolipid to one or more acyl acceptor substrates, wherein the enzyme is obtainable from *Streptomyces* species and includes a lipolytic enzyme comprising an amino acid sequence as shown in SEQ ID No. 4 or an amino acid sequence which has at least 60% identity thereto. The enzyme may be encoded by a nucleic acid selected from the group consisting of: a) a nucleic acid comprising a nucleotide sequence shown in SEQ ID No. 3; b) a nucleic acid which is related to the nucleotide sequence of SEQ ID No. 3 by the degeneration of the genetic code; and c) a nucleic acid comprising a nucleotide sequence which has at least 70% identity with the nucleotide sequence shown in SEQ ID No. 3.

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PROTEIN

FIELD OF INVENTION

The present invention relates to a novel lipolytic enzyme, in particular a novel lipolytic
5 enzyme, and nucleotide sequences encoding same. The present invention also relates
to methods of production of the novel lipolytic enzyme and to uses thereof. The
present invention also relates to methods and uses of a lipolytic enzyme.

TECHNICAL BACKGROUND

10

The beneficial use of lipolytic enzymes active on glycolipids in bread making was
taught in EP 1 193 314. It was taught that the partial hydrolysis products the lyso-
glycolipids were found to have very high emulsifier functionality. However, the
enzymes taught in EP 1 193 314 were also found to have significant non-selective
15 activity on triglycerides which resulted in unnecessarily high free fatty acid.

A lipolytic enzyme from *Fusarium oxysporum* having phospholipase activity has been
taught in EP 0 869 167. This lipolytic enzyme has high triacylglyceride hydrolysing
(lipase) activity. This enzyme is now sold by Novozymes A/S (Denmark) as Lipopan
20 F™.

WO02/00852 discloses five lipase enzymes and their encoding polynucleotides,
isolated from *Fusarium venenatum*, *F. sulphureum*, *Aspergillus berkeleyanum*, *F.*
culmorum and *F. solani*. All five enzymes are described as having triacylglycerol
25 hydrolysing activity, phospholipase and galactolipase activity.

Lipolytic enzyme variants, with specific amino acid substitutions and fusions, have
been produced; some of which have an enhanced activity on the polar lipids compared
to the wildtype parent enzymes. WO01/39602 describes such a variant, referred to as
30 SP979, which is a fusion of the *Thermomyces lanuginosus* lipase, and the *Fusarium*
oxysporum lipase described in EP 0 869 167. This variant has been found to have a

significantly high ratio of activity on phospholipids and glycolipids compared to triglycerides.

In WO02/094123 it was discovered that by selecting lipolytic enzymes which were
5 active on the polar lipids (glycolipids and phospholipids) in a dough, but substantially not active on triglycerides or 1-mono-glycerides an improved functionality could be achieved.

In co-pending PCT application number PCT/IB2005/000875, wild-type lipolytic
10 enzymes having a higher ratio of activity on polar lipids as compared with triglycerides are taught. However, this document does not teach lipolytic enzymes from *Streptomyces*, *Thermobifida* or *Corynebacterium* species.

Prior to the present invention no lipolytic enzymes having activity or significant
15 activity on glycolipids had been published from *Streptomyces* species. Likewise, no lipolytic enzymes having activity or significant activity on glycolipids had been published from *Thermobifida* species or *Corynebacterium* species. Although lipases, i.e. triacylglycerol hydrolysing enzymes, have been isolated from *Streptomyces* species (see Vujaklija *et al Arch Microbiol* (2002) 178: 124-130 for example), these enzymes
20 have never been identified as having glycolipid hydrolysing activity.

ASPECTS OF THE INVENTION

The present invention is predicated upon the seminal finding of a lipolytic enzyme
25 having significant galactolipid activity from the genus *Streptomyces*. In particular the lipolytic enzyme from the genus *Streptomyces* has significant galactolipid hydrolysing activity and/or significant galactolipid acyltransferase activity, particularly when used in the methods and uses according to the present invention.

30 In addition, the present invention is predicated upon the seminal finding that lipolytic enzymes from the genera *Thermobifida* or *Corynebacterium* have significant galactolipid activity. In particular the lipolytic enzymes from the genera *Thermobifida*

or *Corynebacterium* have significant galactolipid hydrolysing activity and/or significant galactolipid acyltransferase activity, particularly when used in the methods and uses of the present invention.

- 5 In a broad aspect the present invention relates to a lipolytic enzyme capable of hydrolysing at least glycolipids and/or capable of transferring an acyl group from at least a glycolipid to one or more acyl acceptor substrates, wherein the enzyme is obtainable, preferably obtained, from *Streptomyces* species.
- 10 In a further aspect the present invention relates to a lipolytic enzyme capable of hydrolysing at least galactolipids and/or capable of transferring an acyl group from at least a galactolipid to one or more acyl acceptor substrates, wherein the enzyme is encoded by a nucleic acid selected from the group consisting of:
- a) a nucleic acid comprising a nucleotide sequence shown in SEQ ID No. 3;
 - 15 b) a nucleic acid which is related to the nucleotide sequence of SEQ ID No. 3 by the degeneration of the genetic code; and
 - c) a nucleic acid comprising a nucleotide sequence which has at least 70% identity with the nucleotide sequence shown in SEQ ID No. 3.
- 20 The present invention yet further provides a lipolytic enzyme comprising an amino acid sequence as shown in SEQ ID No. 4 or an amino acid sequence which has at least 60% identity thereto.

In another aspect the present invention provides a lipolytic enzyme capable of
25 hydrolysing at least a galactolipid and/or capable of transferring an acyl group from at least a galactolipid to one or more acyl acceptor substrates, wherein the enzyme comprises an amino acid sequence as shown in SEQ ID No. 4 or an amino acid sequence which has at least 60% identity thereto.

30 In a further aspect the present invention provides a nucleic acid encoding a lipolytic enzyme comprising an amino acid sequence as shown in SEQ ID No. 4 or an amino acid sequence which has at least 60% identity therewith.

SEQ ID No. 3 is shown in Figure 3 and SEQ ID No. 4 is shown in Figure 4.

The present invention yet further provides a nucleic acid encoding a lipolytic enzyme,
5 which nucleic acid is selected from the group consisting of:

- a) a nucleic acid comprising a nucleotide sequence shown in SEQ ID No. 3;
- b) a nucleic acid which is related to the nucleotide sequence of SEQ ID No. 3 by the
degeneration of the genetic code; and
- c) a nucleic acid comprising a nucleotide sequence which has at least 70% identity
10 with the nucleotide sequence shown in SEQ ID No. 3.

The present invention yet further provides the use of a lipolytic enzyme according to
the present invention in a substrate (preferably a foodstuff) for preparing a lyso-
glycolipid, for example digalactosyl monoglyceride (DGMG) or monogalactosyl
15 monoglyceride (MGMG) by treatment of a glycolipid (e.g. digalactosyl diglyceride
(DGDG) or monogalactosyl diglyceride (MGDG)) with the lipolytic enzyme according
to the present invention to produce the partial hydrolysis product, i.e. the lyso-
glycolipid.

20 In a further aspect, the present invention provides the use of a lipolytic enzyme
according to the present invention in a substrate (preferably a foodstuff) for preparing
a lyso-phospholipid, for example lysolecithin, by treatment of a phospholipid (e.g.
lecithin) with the enzyme according to the present invention to produce a partial
hydrolysis product, i.e. a lyso-phospholipid.

25

In one broad aspect the present invention relates to a method of preparing a foodstuff
the method comprising admixing a lipolytic enzyme of the present invention with one
or more ingredients of the foodstuff.

30 Another broad aspect of the present invention relates to a method of preparing a baked
product from a dough, the method comprising admixing a lipolytic enzyme of the
present invention with the dough.

There is also provided a method of preparing a lipolytic enzyme according to the present invention, the method comprising transforming a host cell with a recombinant nucleic acid comprising a nucleotide sequence coding for the lipolytic enzyme, the host cell being capable of expressing the nucleotide sequence coding for the polypeptide of the lipolytic enzyme, cultivating the transformed host cell under conditions where the nucleic acid is expressed and harvesting the lipolytic enzyme.

In a further aspect the present invention relates to the use of a lipolytic enzyme in accordance with the present invention in the bioconversion of polar lipids (preferably glycolipids) to make high value products, such as carbohydrate esters and/or protein esters and/or protein subunit esters and/or a hydroxy acid ester.

Another aspect of the present invention relates to the use of a lipolytic enzyme in accordance with the present invention in a process of enzymatic degumming of vegetable or edible oil, comprising treating said edible or vegetable oil with said lipolytic enzyme so as to hydrolyse a major part of the polar lipids.

A further aspect of the present invention relates to the use of a lipolytic enzyme in accordance with the present invention in a process comprising treatment of a phospholipid so as to hydrolyse fatty acyl groups.

The present invention yet further relates to an immobilised lipolytic enzyme in accordance with the present invention.

Another aspect of the present invention relates to a method of preparing a lysoglycolipid comprising treating a substrate comprising a glycolipid with at least one lipolytic enzyme to produce said lysoglycolipid, wherein said lipolytic enzyme has glycolipase activity and wherein said lipolytic enzyme is obtainable from one of the following genera: *Streptomyces*, *Corynebacterium* and *Thermobifida*.

A further aspect of the present invention relates to a method of preparing a lysophospholipid comprising treating a substrate comprising a phospholipid with at

least one lipolytic enzyme to produce said lysophospholipid, wherein said lipolytic enzyme has phospholipase activity and wherein said lipolytic enzyme is obtainable from one of the following genera: *Streptomyces*, *Corynebacterium* and *Thermobifida*.

5 Another aspect of the present invention relates to a method of enzymatic degumming of vegetable or edible oil, comprising treating said edible or vegetable oil with a lipolytic enzyme obtainable from one of the following genera: *Streptomyces*, *Corynebacterium* and *Thermobifida* capable of hydrolysing a major part of the polar lipids.

10

The present invention further relates to a method of bioconversion of polar lipids to make high value products comprising treating said polar lipids with a lipolytic enzyme obtainable from one of the following genera: *Streptomyces*, *Corynebacterium* and *Thermobifida* to produce said high value products, wherein said lipolytic enzyme is capable of hydrolysing said polar lipids.

15

Another aspect of the present invention relates to a method of preparing a foodstuff comprising admixing at least one lipolytic enzyme with one or more ingredients of a foodstuff wherein said lipolytic enzyme is capable of hydrolysing a glycolipid and/or a phospholipid present in or as at least one of said ingredients and wherein said lipolytic enzyme is obtainable from one of the following genera: *Streptomyces*, *Corynebacterium* and *Thermobifida*.

20

A further aspect of the present invention relates the use of a lipolytic enzyme in a substrate for preparing a lysophospholipid wherein said lipolytic enzyme has phospholipase activity and wherein said lipolytic enzyme is obtainable from one of the following: *Streptomyces*, *Corynebacterium* and *Thermobifida*.

25

The present invention additionally relates to the use of a lipolytic enzyme obtainable from one of the following genera: *Streptomyces*, *Corynebacterium* and *Thermobifida* for enzymatic degumming of vegetable or edible oil so as to hydrolyse a major part of the polar lipids.

30

Another aspect of the present invention relates to the use of a lipolytic enzyme obtainable from one of the following genera: *Streptomyces*, *Corynebacterium* and *Thermobifida* in a process comprising treatment of a phospholipid so as to hydrolyse
5 fatty acyl groups.

A further aspect of the present invention relates to use of a lipolytic enzyme in the bioconversion of polar lipids to make high value products, wherein said lipolytic enzyme is capable of hydrolysing said polar lipids and wherein said lipolytic enzymes
10 is obtainable from one of the following genera: *Streptomyces*, *Corynebacterium* and *Thermobifida*.

A further aspect of the present invention relates to the use of a lipolytic enzyme obtainable from one of the following genera: *Streptomyces*, *Corynebacterium* and
15 *Thermobifida* in the preparation of a foodstuff, wherein said lipolytic enzyme is capable of hydrolysing a glycolipid and/or a phospholipid.

Aspects of the present invention are presented in the claims and in the following
20 commentary.

Other aspects concerning the nucleotide sequences which can be used in the present invention include: a construct comprising the sequences of the present invention; a vector comprising the sequences for use in the present invention; a plasmid comprising the sequences for use in the present invention; a transformed cell comprising the
25 sequences for use in the present invention; a transformed tissue comprising the sequences for use in the present invention; a transformed organ comprising the sequences for use in the present invention; a transformed host comprising the sequences for use in the present invention; a transformed organism comprising the sequences for use in the present invention. The present invention also encompasses
30 methods of expressing the nucleotide sequence for use in the present invention using the same, such as expression in a host cell; including methods for transferring same.

The present invention further encompasses methods of isolating the nucleotide sequence, such as isolating from a host cell.

Other aspects concerning the amino acid sequence for use in the present invention include: a construct encoding the amino acid sequences for use in the present invention; a vector encoding the amino acid sequences for use in the present invention; a plasmid encoding the amino acid sequences for use in the present invention; a transformed cell expressing the amino acid sequences for use in the present invention; a transformed tissue expressing the amino acid sequences for use in the present invention; a transformed organ expressing the amino acid sequences for use in the present invention; a transformed host expressing the amino acid sequences for use in the present invention; a transformed organism expressing the amino acid sequences for use in the present invention. The present invention also encompasses methods of purifying the amino acid sequence for use in the present invention using the same, such as expression in a host cell; including methods of transferring same, and then purifying said sequence.

For the ease of reference, these and further aspects of the present invention are now discussed under appropriate section headings. However, the teachings under each section are not necessarily limited to each particular section.

DETAILED DISCLOSURE OF THE INVENTION

Suitably, the lipolytic enzyme for use in the methods and uses according to the present invention may be a lipolytic enzyme comprising any one of the amino acid sequences shown as SEQ ID No. 4, 5, 7, 8, 12, 14 or 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, or encoded by any one of the nucleotide sequences shown as SEQ ID No. 3, 6, 9, 13, 15 or 17 or a nucleotide sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

Preferably, the lipolytic enzyme for use in the methods and uses according to the present invention is a lipolytic enzyme capable of hydrolysing at least galactolipids and/or capable of transferring an acyl group from at least a galactolipid to one or more acyl acceptor substrates, wherein the enzyme is obtainable, preferably obtained, from
5 *Streptomyces* species.

In one embodiment the lipolytic enzyme for use in the methods and uses according to the present invention is preferably a lipolytic enzyme capable of hydrolysing at least galactolipids and/or capable of transferring an acyl group from at least a galactolipid to
10 one or more acyl acceptor substrates, wherein the enzyme is encoded by a nucleic acid selected from the group consisting of:

- a) a nucleic acid comprising a nucleotide sequence shown in SEQ ID No. 3;
- b) a nucleic acid which is related to the nucleotide sequence of SEQ ID No. 3 by the degeneration of the genetic code; and
- 15 c) a nucleic acid comprising a nucleotide sequence which has at least 70% identity with the nucleotide sequence shown in SEQ ID No. 3.

In one embodiment, the lipolytic enzyme for use in the methods and uses according to the present invention is preferably a lipolytic enzyme comprising an amino acid
20 sequence as shown in SEQ ID No. 4 or an amino acid sequence which has at least 60% identity thereto.

In another embodiment the lipolytic enzyme for use in the methods and uses according to the present invention is preferably a lipolytic enzyme capable of hydrolysing at least
25 a galactolipid and/or capable of transferring an acyl group from at least a galactolipid to one or more acyl acceptor substrates, wherein the enzyme comprises an amino acid sequence as shown in SEQ ID No. 4 or an amino acid sequence which has at least 60% identity thereto.

30 Preferably, the lipolytic enzyme for use in the methods and uses according to the present invention is a lipolytic enzyme capable of hydrolysing at least galactolipids and/or capable of transferring an acyl group from at least a galactolipid to one or more

acyl acceptor substrates, wherein the enzyme is obtainable, preferably obtained, from *Thermobifida* species, preferably *Thermobifida fusca*.

5 Preferably, the lipolytic enzyme for use in the methods and uses according to the present invention is a lipolytic enzyme capable of hydrolysing at least galactolipids and/or capable of transferring an acyl group from at least a galactolipid to one or more acyl acceptor substrates, wherein the enzyme is obtainable, preferably obtained, from *Corynebacterium* species, preferably *Corynebacterium efficiens*.

10 In a further embodiment the lipolytic enzyme for use in the methods and uses according to the present invention may be a lipolytic enzyme comprising any one of the amino acid sequences shown as SEQ ID No. 4, 5, 7, 8, 12, 14 or 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, or encoded by any one of the nucleotide sequences shown as SEQ
15 ID No. 3, 6, 9, 13, 15 or 17 or a nucleotide sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

In a further embodiment the lipolytic enzyme for use in the methods and uses according to the present invention may be a lipolytic enzyme comprising any one of
20 amino sequences shown as SEQ ID No. 5, 7, 8, 14 or 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith for the uses described herein.

In a further embodiment the lipolytic enzyme for use in the methods and uses according to the present invention may be a lipolytic enzyme comprising any one of
25 amino sequences shown as SEQ ID No. 5, 7 or 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith for the uses described herein.

30 More preferably in one embodiment the lipolytic enzyme for use in the methods and uses according to the present invention may be a lipolytic enzyme comprising the

amino acid sequence shown as SEQ ID No. 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

5 In another embodiment the lipolytic enzyme for use in the methods and uses according to the present invention may be a lipolytic enzyme comprising the amino acid sequence shown as SEQ ID No.s 12 or 14 or an amino acid sequence which has at least 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

10 In another embodiment the lipolytic enzyme for use in the methods and uses according to the present invention may be a lipolytic enzyme comprising the amino acid sequence shown as SEQ ID No. 8 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

15 In one embodiment the lipolytic enzyme for use in the methods and uses according to the present invention may be a lipolytic enzyme capable of hydrolysing at least galactolipids and/or capable of transferring an acyl group from at least a galactolipid to one or more acyl acceptor substrates, wherein the enzyme is encoded by a nucleic acid selected from the group consisting of:

- 20 a) a nucleic acid comprising a nucleotide sequence shown in SEQ ID No. 3;
- b) a nucleic acid which is related to the nucleotide sequence of SEQ ID No. 3 by the degeneration of the genetic code; and
- c) a nucleic acid comprising a nucleotide sequence which has at least 70% identity with the nucleotide sequence shown in SEQ ID No. 3.

25 In one embodiment the lipolytic enzyme according to the present invention may be a lipolytic enzyme obtainable, preferably obtained, from the *Streptomyces* strains L130 or L131 deposited by Danisco A/S of Langebrogade 1, DK-1001 Copenhagen K, Denmark under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of Patent Procedure at the National Collection of
30 Industrial, Marine and Food Bacteria (NCIMB) 23 St. Machar Street, Aberdeen Scotland, GB on 25 June 2004 under accession numbers NCIMB 41226 and NCIMB 41227, respectively.

Preferably, the lipolytic enzyme according to the present invention acts on at least a glycolipid, such as digalactosyldiglyceride (DGDG) for example. Suitably, the lipolytic enzyme according to the present invention may also act on one or more other polar lipid substrates, such as a phospholipid, for example a lecithin, e.g. phosphatidylcholine.

An alternative way of expressing the term "capable of hydrolysing glycolipids" as used herein would be to say that the lipolytic enzyme has glycolipid hydrolysing activity.

Preferably, the lipolytic enzyme according to the present invention hydrolyses a glycolipid, such as digalactosyldiglyceride (DGDG) for example, and also a phospholipid, such as a lecithin, e.g. phosphatidylcholine.

Preferably the lipolytic enzyme according to the present invention acts on glycolipids such as DGDG or MGDG.

In one aspect the lipolytic enzyme according to the present invention hydrolyses DGDG to DGMG and/or MGDG to MGMG.

In one aspect the lipolytic enzyme according to the present invention hydrolyses lecithin to lysolecithin.

When it is the case that the lipolytic enzyme is capable of transferring an acyl group from at least a glycolipid to a donor substrate, the polar lipid substrate may be referred to herein as the "lipid acyl donor".

In one embodiment, the enzyme according to the present invention which as well as having phospholipase and/or glycolipase activity (generally classified as E.C. 3.1.1.26; E.C. 3.1.1.4 or E.C. 3.1.1.32 in accordance with the Enzyme Nomenclature Recommendations (1992) of the Nomenclature Committee of the International Union

of Biochemistry and Molecular Biology) also has acyltransferase activity (generally classified as E.C. 2.3.1.x), whereby the enzyme is capable of transferring an acyl group from a lipid acyl donor to one or more acceptor substrates, such as one or more of the following: a sterol; a stanol; a carbohydrate; a protein; a protein subunit; glycerol.

5

Lipid acyltransferases and their uses are taught in co-pending International Patent Application number PCT/IB2004/000655. This document is incorporated herein by reference. However, the lipolytic enzymes from the genera *Streptomyces* according to the present invention are not taught in PCT/IB2004/000655.

10

In some aspects, the lipolytic enzyme for use in the methods and/or uses of the present invention may be capable of transferring an acyl group from a polar lipid (as defined herein) to one or more of the following acyl acceptor substrates: a sterol, a stanol, a carbohydrate, a protein or subunits thereof, or a glycerol.

15

For some aspects the "acyl acceptor" according to the present invention may be any compound comprising a hydroxy group (-OH), such as for example, polyvalent alcohols, including glycerol; sterol; stanols; carbohydrates; hydroxy acids including fruit acids, citric acid, tartaric acid, lactic acid and ascorbic acid; proteins or a sub-unit thereof, such as amino acids, protein hydrolysates and peptides (partly hydrolysed protein) for example; and mixtures and derivatives thereof.

20

In some aspects, the "acyl acceptor" according to the present invention may be preferably not water.

25

In one embodiment, the acyl acceptor is preferably not a monoglyceride and/or a diglyceride.

In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to a sterol and/or a stanol.

30

In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to a carbohydrate.

5 In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to a protein or a subunit thereof. Suitably the protein subunit may be one or more of the following: an amino acid, a protein hydrolysate, a peptide, a dipeptide, an oligopeptide, a polypeptide.

10 Suitably in the protein or protein subunit the acyl acceptor may be one or more of the following constituents of the protein or protein subunit: a serine, a threonine, a tyrosine, or a cysteine.

15 When the protein subunit is an amino acid, suitably the amino acid may be any suitable amino acid. Suitably the amino acid may be one or more of a serine, a threonine, a tyrosine, or a cysteine for example.

In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to glycerol.

20 In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to a hydroxy acid.

In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to a polyvalent alcohol.

25

In one aspect, the lipolytic enzyme may, as well as being able to transfer an acyl group from a lipid to a sterol and/or a stanol, additionally be able to transfer the acyl group from a lipid to one or more of the following: a carbohydrate, a protein, a protein subunit, glycerol.

30

The term lecithin as used herein encompasses phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidylglycerol.

- 5 For some aspects, preferably the lipid substrate is at least a glycolipid, such as DGDG for example.

For some aspects, preferably the lipid substrate may be additionally a phospholipid, such as lecithin, for example phosphatidylcholine. Other phospholipid substrates in
10 accordance with the present invention may be one or more of N acyl phosphatidyl ethanolamine (APE) or N acyl lyso-phosphatidyl ethanolamine (ALPE).

Preferably the lipid substrate is a food lipid, that is to say a lipid component of a foodstuff.

15

For some aspects, preferably the lipolytic enzyme according to the present invention is incapable, or substantially incapable, of acting on a triglyceride and/or a 1-monoglyceride and/or 2-monoglyceride.

- 20 In one embodiment the lipolytic enzyme according to the present invention has no activity or no significant activity on triglyceride and/or 1-monoglycerides and/or 2-monoglycerides.

Suitably, the lipid substrate or lipid acyl donor may be one or more lipids present in
25 one or more of the following substrates: fats, including lard, tallow and butter fat; oils including oils extracted from or derived from palm oil, sunflower oil, soya bean oil, safflower oil, cotton seed oil, ground nut oil, corn oil, olive oil, peanut oil, coconut oil, and rapeseed oil. Lecithin from soya, rapeseed or egg yolk is also a suitable lipid substrate. The lipid substrate may be an oat lipid or other plant based material
30 containing galactolipids.

In one aspect the lipid substrate or lipid acyl donor is preferably lecithin (such as phosphatidylcholine) in egg yolk.

For some aspects of the present invention, the lipid may be selected from lipids having
5 a fatty acid chain length of from 8 to 22 carbons.

For some aspects of the present invention, the lipid may be selected from lipids having a fatty acid chain length of from 16 to 22 carbons, more preferably of from 16 to 20 carbons.

10

For some aspects of the present invention, the lipid may be selected from lipids having a fatty acid chain length of no greater than 14 carbons, suitably from lipids having a fatty acid chain length of from 4 to 14 carbons, suitably 4 to 10 carbons, suitably 4 to 8 carbons.

15

Suitably, the lipolytic enzyme according to the present invention exhibits at least glycolipase activity (E.C. 3.1.1.26). Suitably, the lipolytic enzyme according to the present invention may also exhibit phospholipase A2 activity (E.C. 3.1.1.4) and/or phospholipase A1 activity (E.C. 3.1.1.32).

20

For some aspects, the lipolytic enzyme according to the present invention may solely have glycolipase activity (E.C. 3.1.1.26).

For some aspects, the lipolytic enzyme according to the present invention is a
25 galactolipase (E.C. 3.1.1.26). The fact that the enzyme is designated as a galactolipase does not, however, prevent it from having other side-activities, such as activity towards other polar lipids for example.

The terms "glycolipase activity" and "galactolipase activity" as used herein are used
30 interchangeably.

Suitably, for some aspects the lipolytic enzyme according to the present invention may be capable of transferring an acyl group from a glycolipid and/or a phospholipid to one or more acceptor substrates.

- 5 Suitably the acceptor substrate may be one or more of the following substrates: a sterol, a stanol, a carbohydrate, a protein, glycerol.

The term "polar lipids" as used herein means phospholipids and/or glycolipids. In some aspects, the term polar lipids preferably means at least glycolipids.

10

The glycolipase activity; phospholipase activity and/or triacylglycerol lipase activity of an enzyme can be determined using the assays presented hereinbelow.

Determination of galactolipase activity (glycolipase activity assay (GLU-7)):

15

Substrate

0.6% digalactosyldiglyceride (Sigma D 4651), 0.4% Triton-X 100 (Sigma X-100) and 5 mM CaCl₂ was dissolved in 0.05M HEPES buffer pH 7.

Assay procedure:

- 20 400 μ L substrate was added to an 1.5 mL Eppendorf tube and placed in an Eppendorf Thermomixer at 37°C for 5 minutes. At time t= 0 min, 50 μ L enzyme solution was added. Also a blank with water instead of enzyme was analyzed. The sample was mixed at 10x100 rpm in an Eppendorf Thermomixer at 37°C for 10 minutes. At time t=10 min the Eppendorf tube was placed in another thermomixer at 99°C for 10
- 25 minutes to stop the reaction.

Free fatty acid in the samples was analyzed by using the NEFA C kit from WAKO GmbH.

Enzyme activity GLU at pH 7 was calculated as micromole fatty acid produced per minute under assay conditions

30

Determination of phospholipase activity (phospholipase activity assay (PLU-7)):

Substrate

0.6% L- α Phosphatidylcholine 95% Plant (Avanti #441601), 0.4% Triton-X 100 (Sigma X-100) and 5 mM CaCl₂ was dispersed in 0.05M HEPES buffer pH 7.

Assay procedure:

5 400 μ L substrate was added to a 1.5 mL Eppendorf tube and placed in an Eppendorf Thermomixer at 37°C for 5 minutes. At time $t=0$ min, 50 μ L enzyme solution was added. Also a blank with water instead of enzyme was analyzed. The sample was mixed at 10x100 rpm in an Eppendorf Thermomixer at 37°C for 10 minutes. At time $t=10$ min the Eppendorf tube was placed in another thermomixer at 99°C for 10
10 minutes to stop the reaction.

Free fatty acid in the samples was analyzed by using the NEFA C kit from WAKO GmbH.

Enzyme activity PLU-7 at pH 7 was calculated as micromole fatty acid produced per minute under assay conditions

15

Determination of triacylglyceride lipase activity: assay based on triglyceride (tributyryn) as substrate (LIPU):

Lipase activity based on tributyrin is measured according to Food Chemical Codex,
20 Forth Edition, National Academy Press, 1996, p 803. With the modification that the sample is dissolved in deionized water in stead of glycine buffer, and the pH stat set point is 5.5 instead of 7.

1 LIPU is defined as the quantity of enzyme which can liberate 1 micromole butyric acid per min. under assay conditions.

25

In one embodiment, preferably the lipolytic enzyme according to the present invention is a wild-type lipolytic enzyme.

The terms "natural" and "wild type" as used herein mean a naturally-occurring
30 enzyme. That is to say an enzyme expressed from the endogenous genetic code and isolated from its endogenous host organism and/or a heterologously produced enzyme which has not been mutated (i.e. does not contain amino acid deletions, additions or

substitutions) when compared with the mature protein sequence (after co- and post-translational cleavage events) endogenously produced. Natural and wild-type proteins of the present invention may be encoded by codon optimised polynucleotides for heterologous expression, and may also comprise a non-endogenous signal peptide
5 selected for expression in that host.

The term "variant" as used herein means a protein expressed from a non-endogenous genetic code resulting in one or more amino acid alterations (i.e. amino acid deletions, additions or substitutions) when compared with the natural or wild-type sequence
10 within the mature protein sequence.

Preferably, the lipolytic enzyme according to the present invention is obtainable (suitably may be obtained) from a bacterium.

15 Preferably, the lipolytic enzyme according to the present invention may be obtainable (preferably obtained) from *Streptomyces* spp. Preferably, the lipolytic enzyme according to the present invention may be obtainable (preferably obtained) from *Streptomyces* strain L131 or *Streptomyces* strain L130.

20 Preferably, the lipolytic enzyme according to the present invention comprises an amino acid sequence which has at least 70%, preferably at least 75%, preferably at least 80%, preferably at least 90%, preferably at least 95%, preferably at least 98%, preferably at least 99% identity with the amino acid sequence shown as SEQ ID No. 4.

25 Preferably, the nucleic acid encoding the lipolytic enzyme according to the present invention comprises a nucleotide sequence which has at least 75%, preferably at least 80%, preferably at least 85%, preferably at least 90%, preferably at least 95%, preferably at least 98%, preferably at least 99% identity with the nucleotide sequence shown in SEQ ID No. 3.

30

In one embodiment suitably the pH optimum of the enzyme on a galactolipid substrate is about 6-8, preferably about 6.5 to 7.5, more preferably about 7.

Suitably, the lipolytic enzyme according to the present invention may not be inhibited or not significantly be inhibited by lipases inhibitors present in wheat flour. The term “not significantly inhibited” as used herein means that the enzyme is less sensitive to lipase inhibitors present in the wheat flour when compared to an equivalent dosage (PLU) of LipopanF™ (Novozymes A/S, Denmark), as based on the standard phospholipase (PLU-7) assay defined herein.

Suitably, the lipolytic enzyme according to the present invention is capable of hydrolysing at least 10% of the galactolipid diester in the substrate (i.e. in the foodstuff, e.g. dough, for instance) to the monoester. Preferably, the enzyme is capable of hydrolysing at least 20%, more preferably at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the galactolipid diester to the monoester. Suitably, the galactolipid diester may be one or more of MGDG or DGDG and the monoester may be one or more of MGMG or DGMG, respectively.

Suitably, the lipolytic enzyme according to the present invention may be isolated from a fermentation broth of *Streptomyces* strain L131 or *Streptomyces* strain L130.

Suitably, the enzyme may be purified by liquid chromatography.

The amino acid sequence of the purified lipolytic enzyme may be determined by Edman degradation, LC-MS and MALDI-TOF analysis.

Suitably, the enzyme as defined herein may catalyse one or more of the following reactions: interesterification, transesterification, alcoholysis, hydrolysis.

The term “interesterification” refers to the enzymatic catalysed transfer of acyl groups between a lipid donor and lipid acceptor, wherein the lipid donor is not a free acyl group.

The term "transesterification" as used herein means the enzymatic catalysed transfer of an acyl group from a lipid donor (other than a free fatty acid) to an acyl acceptor (other than water).

- 5 As used herein, the term "alcoholysis" refers to the enzymatic cleavage of a covalent bond of an acid derivative by reaction with an alcohol ROH so that one of the products combines with the H of the alcohol and the other product combines with the OR group of the alcohol.
- 10 As used herein, the term "alcohol" refers to an alkyl compound containing a hydroxyl group.

As used herein, the term "hydrolysis" refers to the enzymatic catalysed transfer of an acyl group from a lipid to the OH group of a water molecule. Acyl transfer which
15 results from hydrolysis requires the separation of the water molecule.

The term "foodstuff" as used herein means a substance which is suitable for human and/or animal consumption.

- 20 Suitably, the term "foodstuff" as used herein may mean a foodstuff in a form which is ready for consumption. Alternatively or in addition, however, the term foodstuff as used herein may mean one or more food materials which are used in the preparation of a foodstuff. By way of example only, the term foodstuff encompasses both baked
25 goods produced from dough as well as the dough used in the preparation of said baked goods.

In a preferred aspect the present invention provides a foodstuff as defined above wherein the foodstuff is selected from one or more of the following: eggs, egg-based
30 products, including but not limited to mayonnaise, salad dressings, sauces, ice creams, egg powder, modified egg yolk and products made therefrom; baked goods, including breads, cakes, sweet dough products, laminated doughs, liquid batters, muffins, doughnuts, biscuits, crackers and cookies; confectionery, including chocolate, candies,

caramels, halawa, gums, including sugar free and sugar sweetened gums, bubble gum, soft bubble gum, chewing gum and puddings; frozen products including sorbets, preferably frozen dairy products, including ice cream and ice milk; dairy products, including cheese, butter, milk, coffee cream, whipped cream, custard cream, milk
5 drinks and yoghurts; mousses, whipped vegetable creams, meat products, including processed meat products; edible oils and fats, aerated and non-aerated whipped products, oil-in-water emulsions, water-in-oil emulsions, margarine, shortening and spreads including low fat and very low fat spreads; dressings, mayonnaise, dips, cream based sauces, cream based soups, beverages, spice emulsions and sauces.

10

Suitably the foodstuff in accordance with the present invention may be a "fine foods", including cakes, pastry, confectionery, chocolates, fudge and the like.

In one aspect the foodstuff in accordance with the present invention may be a dough
15 product or a baked product, such as a bread, a fried product, a snack, cakes, pies, brownies, cookies, noodles, snack items such as crackers, graham crackers, pretzels, and potato chips, and pasta.

In a further aspect, the foodstuff in accordance with the present invention may be a
20 plant derived food product such as flours, pre-mixes, oils, fats, cocoa butter, coffee whitener, salad dressings, margarine, spreads, peanut butter, shortenings, ice cream, cooking oils.

In another aspect, the foodstuff in accordance with the present invention may be a
25 dairy product, including butter, milk, cream, cheese such as natural, processed, and imitation cheeses in a variety of forms (including shredded, block, slices or grated), cream cheese, ice cream, frozen desserts, yoghurt, yoghurt drinks, butter fat, anhydrous milk fat, other dairy products. The enzyme according to the present invention may improve fat stability in dairy products.

30

It is particularly advantageous to utilise the enzyme according to the present invention in cheese. Thus, a lipolytic enzyme in accordance with the present invention can

advantageously be used to produce cheese. The lipolytic enzyme catalyses the hydrolysis of phospholipids in the milk which contributes to increased cheese yield. Preferably the lipolytic enzyme according to the present invention may be added to milk (referred to as cheese milk) prior to or during the cheese making process.

5

In another aspect, the foodstuff in accordance with the present invention may be a food product containing animal derived ingredients, such as processed meat products, cooking oils, shortenings.

- 10 In a further aspect, the foodstuff in accordance with the present invention may be a beverage, a fruit, mixed fruit, a vegetable or wine. In some cases the beverage may contain up to 20 g/l of added phytosterols.

In another aspect, the foodstuff in accordance with the present invention may be an
15 animal feed. The animal feed may be enriched with phytosterol and/or phytosterols, preferably with beta-sitosterol/stanol. Suitably, the animal feed may be a poultry feed. When the foodstuff is poultry feed, the present invention may be used to lower the cholesterol content of eggs produced by poultry fed on the foodstuff according to the present invention.

20

In one aspect preferably the foodstuff is selected from one or more of the following: eggs, egg-based products, including mayonnaise, salad dressings, sauces, ice cream, egg powder, modified egg yolk and products made therefrom.

- 25 Preferably the foodstuff according to the present invention is a water containing foodstuff. Suitably the foodstuff may be comprised of 10-98% water, suitably 14-98%, suitably of 18-98% water, suitably of 20-98%, suitably of 40-98%, suitably of 50-98%, suitably of 70-98%, suitably of 75-98%.

- 30 For some aspects, the foodstuff in accordance with the present invention may not be a pure plant derived oil, such as olive oil, sunflower oil, peanut oil, rapeseed oil for instance. For the avoidance of doubt, in some aspects of the present invention the

foodstuff according to the present invention may comprise an oil, but the foodstuff is not primarily composed of oil or mixtures of oil. For some aspects, preferably the foodstuff comprises less than 95% lipids, preferably less than 90% lipids, preferably less than 85%, preferably less than 80% lipids. Thus, for some aspects of the present invention oil may be a component of the foodstuff, but preferably the foodstuff is not an oil *per se*.

The advantages of using a lipolytic enzyme capable of transferring an acyl group in food applications is taught in patent applications WO2004/064987, WO2004/064537, PCT/IB2004/004374 and GB0513859.9 which are incorporated herein by reference.

The production of free fatty acids can be detrimental to foodstuffs. Free fatty acids have been linked with off-odours and/or off-flavours in foodstuffs, as well other detrimental effects, including a soapy taste in dairy products such as cheese for instance. Suitably in some embodiments of the present invention the lipolytic enzyme is capable of transferring the fatty acid from the lipid to an acyl acceptor, for example a sterol and/or a stanol. Hence, the overall level of free fatty acids in the foodstuff does not increase or increases only to an insignificant degree. Thus, a lipolytic enzyme capable of transferring an acyl group according to the present invention may provide one or more of the following unexpected technical effects in the production of cheese: a decrease in the oiling-off effect in cheese; an increase in cheese yield; an improvement in flavour; a reduced mal-odour; a reduced "soapy" taste.

The utilisation of a lipolytic enzyme taught herein which can transfer the acyl group to a carbohydrate as well as to a sterol and/or a stanol is particularly advantageous for foodstuffs comprising eggs. In particular, the presence of sugars, in particular glucose, in eggs and egg products is often seen as disadvantageous. Egg yolk may comprise up to 1% glucose. In accordance with the present invention this unwanted sugar can be readily removed by "esterifying" the sugar to form a sugar ester.

The presence of diglycerides in edible oils is disadvantageous. In particular, diglycerides in edible oils (in particular palm oil) can lead to a low quality oil. Suitably

in some embodiments of the present invention a lipolytic enzyme taught herein is capable of transferring the fatty acid from the lipid to an acyl acceptor which reduces the level of diglycerides in the oil without increasing or significantly increasing the level of free fatty acids.

5

A lipolytic enzyme taught herein is able to hydrolyse a major part of the phospholipids in an edible or vegetable oil. This is highly advantageous in the enzymatic degumming of vegetable or edible oils. Suitably in some embodiments of the present invention the lipolytic enzyme may be capable of transferring the fatty acid from the
10 lipid to an acyl acceptor. Hence, advantageously the overall level of free fatty acids in the oil does not increase or increases only to an insignificant degree. The production of free fatty acids can be detrimental in the edible oil. Preferably, the method according to the present invention results in the degumming of an edible oil wherein the accumulation of free fatty acids is reduced and/or eliminated.

15

The claims of the present invention are to be construed to include each of the foodstuffs listed above.

In some of the applications mentioned herein, particularly the food applications, such
20 as the bakery applications, the lipolytic enzyme according to the present invention may be used with one or more conventional emulsifiers, including for example monoglycerides, diacetyl tartaric acid esters of mono- and diglycerides of fatty acids, sugar esters, sodium stearoyl lactylate (SSL) and lecithins.

25 In addition or alternatively, the enzyme according to the present invention may be used with one or more other suitable food grade enzymes. Thus, it is within the scope of the present invention that, in addition to the lipolytic enzyme of the present invention, at least one further enzyme may be added to the baked product and/or the dough. Such further enzymes include starch degrading enzymes such as endo- or exoamylases,
30 pullulanases, debranching enzymes, hemicellulases including xylanases, cellulases, oxidoreductases, e.g. glucose oxidase, pyranose oxidase, sulfhydryl oxidase or a carbohydrate oxidase such as one which oxidises maltose, for example hexose oxidase

(HOX), lipases, phospholipases and hexose oxidase, proteases, and acyltransferases (such as those described in PCT/IB2004/000575 for instance).

The present invention encompasses food enzyme compositions, including bread and/or
5 dough improving compositions comprising the enzyme according to the present
invention, and optionally further comprising another enzyme, such as one or more
other suitable food grade enzymes, including starch degrading enzymes such as endo-
or exoamylases, pullulanases, debranching enzymes, hemicellulases including
xylanases, cellulases, oxidoreductases, e.g. glucose oxidase, pyranose oxidase,
10 sulfhydryl oxidase or a carbohydrate oxidase such as one which oxidises maltose, for
example hexose oxidase (HOX), lipases, phospholipases and hexose oxidase, proteases
and acyltransferases (such as those described in PCT/IB2004/000575 for instance).

In some applications mentioned herein, particularly in food applications, such as the
15 bakery applications, the lipolytic enzyme according to the present invention may be
added in combination or sequentially with one or more enzyme substrates. By way of
example only, the lipolytic enzyme according to the present invention may be added
together with one or more polar lipid substrates and/or one or more acyl acceptor
substrates.

20

In some applications mentioned herein, particularly in food applications, such as the
bakery applications, the lipolytic enzyme according to the present invention may be
used with one or more hydroxy acids, including for example tartaric acid, citric acid,
lactic acid, succinic acid or ascorbic acid for example.

25

The term "improved properties" as used herein means any property which may be
improved by the action of the lipolytic enzyme of the present invention. In particular,
the use of the lipolytic enzyme according to the present invention results in one or
more of the following characteristics: increased volume of the baked product;
30 improved crumb structure of the baked product; anti-staling properties in the baked
product; increased strength, increased stability, reduced stickiness and/or improved
machinability of the dough.

The improved properties are evaluated by comparison with a dough and/or a baked product prepared without addition of the lipolytic enzyme according to the present invention.

5

The term "baked product" as used herein includes a product prepared from a dough. Examples of baked products (whether of white, light or dark type) which may advantageously produced by the present invention include one or more of the following: bread (including white, whole-meal and rye bread), typically in the form of loaves or rolls, steam buns, French baguette-type bread, pita bread, tacos, corn tortilla, wheat tortilla, cakes, pancakes, biscuits, crisp bread, pasta, noodles and the like.

10

The dough in accordance with the present invention may be a leavened dough or a dough to be subjected to leavening. The dough may be leavened in various ways such as by adding sodium bicarbonate or the like, or by adding a suitable yeast culture such as a culture of *Saccharomyces cerevisiae* (baker's yeast).

15

The present invention further relates to the use of the lipolytic enzyme in accordance with the present invention to produce a pasta dough, preferably prepared from durum flour or a flour of comparable quality.

20

The lipolytic enzyme according to the present invention is suitable for use in the enzymatic degumming of vegetable or edible oils. In processing of vegetable or edible oil the edible or vegetable oil is treated with lipolytic enzyme according to the present invention so as to hydrolyse a major part of the polar lipids (e.g. phospholipid). Preferably, the fatty acyl groups are hydrolysed from the polar lipids. The degumming process typically results in the reduction of the content of the polar lipids, particularly of phospholipids, in an edible oil due to hydrolyse of a major part (i.e. more than 50%) of the polar lipid, e.g. phospholipid. Typically, the aqueous phase containing the hydrolysed polar lipid (e.g. phospholipid) is separated from the oil. Suitably, the edible or vegetable oil may initially (pre-treatment with the enzyme according to the present invention) have a phosphorus content of 50-250 ppm.

25

30

In one embodiment, the present invention relates to the use of the lipolytic enzyme in accordance with the present invention in the bioconversion of polar lipids (preferably glycolipids) to make high value products, such as carbohydrate esters and/or protein esters and/or protein subunit esters and/or a hydroxy acid ester. The use of a lipolytic enzyme, particularly a lipolytic enzyme capable of transferring acyl groups from a polar lipid substrate (preferably a glycolipid) to an acyl acceptor, in the bioconversion of polar lipids and the advantages thereof is detailed in PCT/IB2004/004374 incorporated herein by reference.

10

In one embodiment the lipolytic enzyme for use in the methods of the present invention may be immobilised. When it is the case that the enzyme is immobilised the admixture comprising an acyl donor, optionally an acyl acceptor, and optionally water may be passed through a column for example comprising the immobilised enzyme. By immobilising the enzyme it is possible to easily reuse it.

15

Suitably, the immobilised enzyme may be used in a flow reactor or in a batch reactor containing a reaction mixture which comprises a lipid acyl donor and optionally an acyl acceptor dissolved in water. When the acyl acceptor is present the donor and acceptor are in a two-phase system or an emulsion. The reaction mixture may be optionally stirred or sonicated. Once the reaction has reached equilibrium for example, the reaction mixture and the immobilised enzyme may be separated. Suitably, the reaction product may be fractionated for example by hydrophobic interaction chromatography, crystallisation or high vacuum distillation.

20

Immobilised lipid acyl transferase can be prepared using immobilisation techniques known in the art. There are numerous methods of preparing immobilised enzymes, which will be apparent to a person skilled in the art (for example the techniques referred to in EP 0 746 608; or Balcao V.M. et al *Enzyme Microb Technol.* 1996 May 1; 18(6):392-416; or Retz et al *Chem Phys Lipids* 1998 June:93(1-2) : 3-14; Bornscheuer et al *Trends Biotechnol.* 2002 Oct; 20(10):433-7; Plou et al *Biotechnology* 92 (2002) 55-66; Warmuth et al 1992 *Bio Forum* 9, 282-283; Ferrer et

25

30

al 2000 J. Chem Technol. Biotechnol. 75, 1-8; or Christensen et al 1998 Nachwachsende Rohstoff 10, 98-105; Petersen and Christensen 2000 Applied Biocatalysis Harwood Academic Publishers, Amsterdam (each of which is incorporated herein by reference).

5

Techniques which may be used herein include covalent coupling to Eupergit C, adsorption on polypropylene and silica-granulation for example.

LIPOLYTIC ENZYMES IN ACCORDANCE WITH THE PRESENT INVENTION

10

The lipolytic enzyme for use in accordance with the present invention and/or the methods described herein is preferably a lipolytic enzyme capable of hydrolysing at least galactolipids and/or capable of transferring an acyl group from at least a galactolipid to one or more acyl acceptor substrates, wherein the enzyme is encoded by a nucleic acid selected from the group consisting of:

15

- d) a nucleic acid comprising a nucleotide sequence shown in SEQ ID No. 3;
- e) a nucleic acid which is related to the nucleotide sequence of SEQ ID No. 3 by the degeneration of the genetic code; and
- f) a nucleic acid comprising a nucleotide sequence which has at least 70% identity with the nucleotide sequence shown in SEQ ID No. 3.

20

Preferably, the lipolytic enzyme used in accordance with the present invention and/or in the methods described herein is a lipolytic enzyme comprising an amino acid sequence as shown in SEQ ID No. 4 or an amino acid sequence which has at least 60% identity thereto.

25

However, the lipolytic enzyme for use in accordance with the present invention and/or in the methods of the present invention may be any lipolytic enzyme obtainable from *Streptomyces* species which is capable of hydrolysing at least a galactolipid and/or capable of transferring an acyl group from a galactolipid to one or more acyl acceptor substrates.

30

Suitable lipolytic enzymes having galactolipase activity for use in accordance with the present invention and/or in the methods of the present invention may comprise any one of the following amino acid sequences and/or be encoded by the following nucleotide sequences:

5

Thermobifida\fusca GDSx 548 aa

SEQ ID No. 5

10 ZP 00058717

1 mlphpagerg evgaffallv gtpqdrirl echetrplrg rcgcgerrvp ptilpgdgl
 61 citsstrdae twrkhkqpr pdggfrphlg vgcilaggs pgvlwvcreg crfevcrndt
 121 pglstrngd sspfragws lppkcgelsq sarktpavpr yslrtdrpd gprgrfvsg
 181 praatrrif lgpalvlt altlvavpt gretlwmwc eatqdwclgv pvdsrgqpa
 15 241 dgefillspv qaatwgnyya lgdsyssgdg ardyypgtav kggcwsana ypelvaeayd
 301 faghlsflac sgqrgyamld aidevgsqld wnsphslvt igiggndlgf stvlkcmvr
 361 vplldskact dqedairkm akfettfeel isevtrapd anilvgypr ifpeeptgay
 421 yltasnrw lneltqefnq qlaeavavhd eeiaasggvg svefvdvyha lgheigsde
 481 pwwngvqlrd latgvtvdrs tthpnaaghr avgervieqi etgprplya favvagatv
 20 541 dtlagevg

SEQ ID No. 6

1 ggtggtgaac cagaacaccc ggtcgtcggc gtgggctcc aggtgcaggt gcaggttct
 25 61 caactgctcc agcaggatgc cggcgtggcc gtgcacgatg gccttgggca ggcctgtgt
 121 ccccgacgag tacagcacc atagcggatg gtcgaacggc agcggggga actccagtc
 181 cgcgcctcg ccgcggtct cgaactccgc ccaggacagg gtgtcggcga caggccgca
 241 gcccaggatc ggcaggacga cgggtgtctg caggctgggc atgcccgtcg gcagggtct
 301 gagcacgta cggcgttga agtccctacc gccgtagcgg tagccgtcca cggccagcag
 361 cacttccgt tcatctgca cgaaccggc gaggacgctg cgcacccga agtcggggg
 421 acaggacgac caggctcgc ccatcgggc gcaggcagg aatgcggccg tgcctcggc
 481 gatgtcggc aggtaggcca cgaccggc gccggggccc accccagggc tgcggagggc
 541 cgcagcgtc gggcgggtg ggtccgag ttcctccag gtccactcg tcaacggccg
 601 gatctggac gctgcccga tgcaccggc tcatgggtca cggctcggga agatgtct
 35 661 ggcgtagtg aggggtggc cggggaacca gacggcggc ggcctggct cggagggcag
 721 cactgtgtg tacgggtgg cggcgcgac ccggtatgac tcccagatc cggaccagaa
 781 tcttcgagg tccgtaccg accagcggc cagtcctcg tagtccgtg cgtccacac
 841 gcggtgctc cgcaccagc ggtgaaacg ggtgaggtg ggcgttct tgcctctc
 901 gtcgggact cacaggatc gcggtcggc ctgagtgat atgaaacgc acccttct
 40 961 ggacgggtg gatcgggtg gctcgggtg cctcccctaa cgtccccgg tgcggagtg
 1021 ttgicacca catctagcag cgggacgc gaaaccgtat ggagaaaaca cclacaacc
 1081 cggccggacg gtgggttcg gccacacta ggggtcgggt gcctgctgc cggcagggc
 1141 aglccccggg tgcgtgtg cggcgggag ggcgtgctc tggaggtg cggcgggac
 1201 adccccggc tcagccgac ccgcaacgg gacagttct cctctccg gctggatgg
 45 1261 tccctccc cgaatgctg cagatctcc cagtcagccc ggaacaccc cgtgtgccc
 1321 aggtactct tgcctgaac agacaggcc gacggctcc gggggaggt tggggcagc
 1381 ggaccagtg cggcagacc acgacggtg ttcctcgta tcccgtct tctactgtg
 1441 acagcgtca cgtggtct ggtgtccc acggggcgc agacgctg ggcaltgtg
 1501 tgtgagcca cccaggact gtccctggg gtccggctg actcccggc acagcctgc
 50 1561 gaggacggc agltctgt cttctccg gtccaggcag cagcctggg gaactattc
 1621 gcgtcggg atctgact tccggggac gggggcccg actactatc cggcaccgc
 1681 gtaagggc gttctggc gtccgtaac gcctatccg agctgtcgc cgaagcctc

1741 gacitgccc gacactgtc gtctcggcc tgcagcgcc agcgcggcta cgccaigt
 1801 gacgctatcg acgaggtcgg ctgcagctg gactggaact cccctcacac gtcgctggg
 1861 acgatcggga tcggcgcaaa cgaictgggg ttctccacgg ttitgaagac ctgcatggg
 1921 cgggtgccgc tctggacag caaggcgtg acggaccagg aggacgctat ccgcaagcgg
 5 1981 atggcgaaat tcgagacgac gttgaagag ctatcagcg aagtgcgac ccgctcggc
 2041 gacgcccga tcttctgtt gggctacccc cggatttct cggaggaacc gaccggcgcc
 2101 tactacacgc tgaccgcgag caaccagcgg tggctcaacg aaaccattca ggagttcaac
 2161 cagcagctcg ccgaggctgt cgcggctcac gacgaggaga ttgccgcgtc gggcggggg
 2221 ggcagcgtgg agttcgtgga cgtctaccac gcgttggacg gccacgagat cggctcggac
 10 2281 gagccgtggg tgaacggggg gcaagtgcgg gacctcgcca ccgggggtgac tgggaccgc
 2341 agtacctcc accccaacgc cgcggggcag cgggcccgtc gtagcgggt caicgagcag
 2401 atcgaacccg gcccgggcgg tccctctat gccacttcc cgggtggggc gggggcgacc
 2461 gtggacactc tcgcgggcga ggtgggggga ccgggctac cgtccggccc gcaggtctg
 2521 gagcactcgc gcgatctgtt ccaactgcca gtgcagttcg tctcgggga tgaccagcg
 15 2581 cggggagagc cggatcgtg agccgtgctg gctttgacg agcacacccc gctcagggag
 2641 ccgttcgac agttctctc cgggtggcag agtcgggtcg acgtgatcc cagcccacag
 2701 gccgatctg cgggcccga ccacgcccgt gccgaccagt tggctgaggc gggcgcgag
 2761 cacggggggc agggcgcgga catggctcag gtaagggccg tgcgggacga ggcaccac
 2821 ggcagtgcc accgcgacgg cgaaggcggt gccgccaag gtgctccgt gctggccggg
 20 2881 gggatcacg tcaagactt ccgcgtgcc taccgccc gccacgggca ggaigccgg
 2941 gccacgctc ttccgaaca gtagatatt ggcgtcgacl ccgctgtgtt cgcaggccc
 //

Thermobifida\fusca\ - GDSx

25

SEQ ID No. 7

1 vsgpraatr rrtgipal vltaltvl avptgrethw mwceatqdw cigvpvdsrg
 61 qpaedgell lsvvqaatwg nyyalgdsys sgdgardyyp gtavkggcwr sanaypelva
 121 eaydfaghl flacsqrgy amidaidevg sqldwnsphi slvtigggd dlfstvlkt
 181 cmrvrpilds kacitdqedai rkrmakfett feelisevri rapdarilv gyprifpeep
 241 tgayytlas nqrwlnetiq efnqqlaev avhdeeiaas gvgvsvefvd vyhaldghei
 301 gsdepwvngv qlrdlatgt vdrstfhpna aghravgerv iqietgpgp phtafavva
 361 gatvdlage vg

35

Corynebacterium\efficiens\ GDSx 300 aa

SEQ ID No. 8

1 mrttviaasa lllagcadg areetagapp gessggiree gaeastsitd vyalgdsya
 61 amggrdqpr gepfcrssg nypellhaev tdlcggavt gdlleprtig ertpaqvda
 121 ltedtlvll siggndltg evagciren agenaddcud lletigeql dqlppqldr
 181 heairdragd aqvvtgylp lvsagdcpel gdvseadrw aveltgqine tvreaaerhd
 241 alfvipddad ehiscappqk rwadiqqqt dayplhpts gheamaaavr dalglepvqp
 //

45

SEQ ID No. 9

50

1 ttctgggggt ttatgggggt gttatcggct cgtcctgggt ggalcccgc aggtggggta
 61 ttacaggggg acttttgtt ccaacagccg agaatgagt cctgagcgg tgggaatgag

121 gtgggaggg ctgtctgcc atgagggggc ggaggggctct gtggtgccc gcgaccccc
 181 gccccgtga gcggtgaatg aaatccggct gtaatcagca tcccgtgcc accccgtcgg
 241 ggaggtcagc gcccggagtg tctacgcagt cggatcctct cggactcggc calgtctcg
 301 gcagcatcg gctcccgggt ctggcgctc ctggcctgt ctgctctg tccctggaag
 5 361 gcgaaatgat caccggggag tgatacaccg gtggtctcat cccggatgcc cacttcggcg
 421 ccatccggca atccgggcag ctccgggtgg aagtaggtgg catccgatgc gtcggigacg
 481 ccatagtggg cgaagatctc atcctgtctg aggggtctca ggccactctc cggatcgata
 541 tcgggggct cctgaatggc gtcctgtctg aaaccgaggt gcagctgtg ggctccaat
 601 ttcgaccac ggagcgggac gaggctggaa tgacggccga agagcccgtg gggacctca
 10 661 acgaaggtgg gtagtcccgt gicatcattg aggaacacgc cctccaccgc acccagctg
 721 tggccggagt tctctaggc gctggcatcc agaagggaaa cgatctcata ttgtcggtg
 781 tgcacagaca tgatctct tgcctgtcgg tgcctgttac taccacggta gggctgaatg
 841 caactgtat ttctgtta tttaggaat tggiccatat cccacaggct ggctgggtc
 901 aaatcgtat caagtaatc ctgtcacaca aaatgggtg tgggagccct ggctcgggt
 15 961 ccgtgggagg cgcctgcc cgcaggatcg tcggcatcgg cggatctggc cggtaccccg
 1021 cggtaataa aatcattctg taacctcat cacggtgtgt tttaggtatc cgcctctc
 1081 gtcctgacc cgtcccggc gcgaggagc ccgagggtg cggtagacag gggagacgig
 1141 gacacatga ggacaacgti catcgcagca agcgcattac tctctctgc cggatgcgcg
 1201 galggggccc gggaggagac cgcgggtgca ccgcccggtg agtctccgg gggcatccgg
 20 1261 gaggaggggg cggaggcgtc gacaagcatc accgacgtct acatcgcctt cggggattcc
 1321 tatcggcga tggcggggcg ggalcagccg ttacggggtg agccgtctg cctgcctcg
 1381 tccgtaait acccgaact cctccacgca gaggtcaccg atctcactc ccagggggcg
 1441 gtgaccggg atctgtctga acccaggagc ctgggggagc gcacgtctcc ggcgagggtg
 1501 gatgcctga cggaggacac caccctgtc acctctcca tcgggggcaa tgactcggg
 25 1561 ttcggggagg tggcgggatg calccgggaa cggatcggcg gggagaacgc tgatgatgc
 1621 gtggacctgc tgggggaaac catcggggag cagctcgtac agctcccc gcagctggac
 1681 cgcgtgcacg aggtatccg ggaccgcgcc ggggacgcgc aggtgtgtgt caccggtac
 1741 ctgcccctg tctctcggg ggaactcggc gaactgggg atgtctccga ggcggatct
 1801 cgttggcgg ttgagctgac cgggcagatc aacgagaccg tgcgcgagcc ggcggaacga
 30 1861 cacgatgcc tcttgtct gcccgacgat gccgatgagc acaccagttg tgcacccca
 1921 cagcagcct gggcgatat ccaggggcaa cagaccgatg cctatccgti gcacccgacc
 1981 tccgcccggc atgaggcgtg gcccggccc gtcggggagc cgtcgggctt ggaaccggct
 2041 cagccgtagc gcccggcgcg cgtctgtcga cgaccaacc atgcaggcti gcagtcacat
 2101 ccgcacatag cgcgcgggg cgaatgggta cgcacatag aggatgagcc cgaatccgac
 35 2161 galgatgagc agcacactgc cgaagggtg ttcccggagg gtcgcagag ccgagtcag
 2221 acctcggcc tgcctcggat catgggcca accggcgtat acgatcaaca ccccaggat
 2281 cccgaaggcg ataccaggg cgacataacc ggctgtccg gtagatga tgcgggtcc
 2341 gacctgccd gacccgcac ccgctccag atctcccgg aaatcccgg tggccccct
 2401 ccagagggtg tagacaccg ccccagctac caccagccc ggcaccaca ccagcaccac
 40 2461 acccagggt tggatagga cggggcggg gacatcgtg gcggctccc catcggaggt
 2521 gctgcccgc cgggcgaagg tggaggtgt caccgccagg gagaagtaga ccatggccat
 2581 gaccgcccc tggccctt cctgagggtc ctgcccggc agcagctggc tcaatgcca
 2641 gagtcccagg gccgcccagg cgaatgagcc aaccacagg aggaactgcc caccggagc
 2701 ctcccgatg gtggccagg cactgaatt cgaggctca tcaccgaac cgcgggatcc
 45 2761 agtggcgatg cgcaccgca tccaccgat gaggatgtc agtatgcca ggacaatgaa
 2821 accacctg gccagggtg tcagcgcgg ggtctctc gctgggtcgg cagcccgtc
 2881 gatctcct ttcgggatc tgggtcgc cttatccata gctccattg aaccgctg
 2941 aggggtggg gccactgtc agggcggatt gtagatgaa ctgtgatgt ccatcaacc

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S.coelicolor\ GDSx 268 aa

5 SEQ ID No. 12

NP 625998.

1 mrrfrlvgl ssvlaagaa lgaataqaa qpaaadgyva lgdsyssvgv agsyissgd
 10 61 ckrstkahpy hwaaahpspt fdfitacsgr lgvlsqqlg pissgtglvs isigndagf
 121 admittcvlq sessclsria taeayvdstl pgkldgvysa isdkapnavh vvigyprfyk
 181 lgticglse tkrtainkas dhntvlaqr aaahgftgd vrtfghel csqspwihsv
 241 nwnigesyh ptaagqsggy lplvngaa

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15 SEQ ID No. 13

1 ccggggcgcc cgtgcaggag cagcagccgg ccgcgatgt cctcgggct cgtctcatic
 20 61 agggcgtcca tcgctcggc gaccggcgcc gtgtagtgg ccgggacct gtcccaggig
 121 ccggggcga tctggcgggt ggtgcggigc gggccgcgc gaggggagac gtaccagaag
 181 ccatcglica cgtctccgg ctgcggctc ggctcgtccg ccgctccgtc cgtcgcctc
 241 ccgagcaact tctcggcag gtcggcgtg gtcgccgtca ccgtgacgtc ggcgccccg
 301 ctccagcgc agatcagcag cgtccagccg tcgcccctcc ccagcgtcgc gctcgggtc
 361 tctcgcggg cgtaccgcag cagcgcgcgc ccgggcggca gcagcgtggc gccggacct
 421 acggggtcga tgttcggcg gtcgagtac ggctgctcac ccgtggcgaa acggccgagg
 481 aacagcgcgt cgacgacgtc ggacggggag tgcctgctc caccgtgag ccggtcggc
 541 agggctcgt gcgggtcac ggacalgtc ccatgctcg gcacccggcc gccgcgtgca
 601 ccgcttcc cgggcacgca cgacggggc tctcgcgcg tctccgtc gaactgaac
 661 gagtgcagc cactctgg catggacct tccagtaac gcgcgtagct gctaccacgg
 721 ttgggcagc aatcctgcta agggaggct catgagacgt tccgactg tggcttct
 781 gagtgcctc gctcgcgc ccggcgccgc cctcaccgg gcagcagacc ccagggcgc
 841 ccaaccgcc gccgcgagc gctatgggc cctcggcgac tctactct ccggggtcgg
 901 agcgggcagc facatcagct cgagcggcga ctgcaagcgc agcaogaagg cccatcccta
 961 cctgtggcg gccgccact cgcclccac gttcagctc accgctgtl ccggcgccc
 1021 tacgggtgat gttctcgc gacagctcg ccgcctcagc tccggaccg gccctgtc
 1081 gatcagcctc ggcggcaac acgcccgtt cgcgacacc atgacacct gttgtctca
 1141 gtcgagagc tctgctgt cgcggtcgc caccgcccag gcgtacgtc actcagcct
 1201 gccggcaag ctgcagcgc tctactggc aatcagcgc aaggcgcga acgcccact
 1261 cgtctcatic ggctaccgc gctctiaaa gctcggcacc acctgcatc gctgtcga
 1321 gaccaagcg acggcgatca acaaggcctc cgaccacct aacaccgtc tcgcccagc
 1381 cgcggccc cagcgttca cctcggcga cgtacgacc acctcaccg gccacgagct
 1441 gtcctcggc agcccctgc tgcacagct caactggctg aacatcggc agtctgacca
 1501 cccaccgcg gccggcact ccggtgcta cctcgggtc ccaaccggc ccgctgacc
 1561 tcaggcggaa ggagaagaag aaggagcga gggagacgag gagtgggagg ccccgcccga
 1621 cggggctccc gtcctcgt cgtctcgt cccggtccg caagtaccg agaacgccac
 1681 cgcgtcgc gtcggcgc cggactccg cactccacg cgcacggc tctcgaacgc
 1741 gccggtgc tctgctgc taccaccac gccctcgt cgcgagcgt cgcgcccga
 1801 cgggaaggac agcgtccgc accccgac ggagaccgc ccgtccgcg taaccacc
 1861 gtgcccacc tccgcccga gcccccgc cgtgaacgc ccgtgaac cgggtgccc
 1921 gtcgtcggc ggcggacag ccccgagla ggggtcgc gagcccacca cgtcactc
 1981 caccgactc gctcggggc

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S. avermitilis\ GDSx 269 aa

SEQ ID No. 14

NP_827753.

5 1 mrrsnitayv tslllavgca ltgaataqas paaaatgyva lgdsyssvgv agsylvssgd
61 ckrsskaypy lwqaahspss fsfmaccsgar tgdvianqlg tlnsstglvs liggndagf
121 sdvmttcvlq sdsacksrin takayvdsti pgqldsvyfa istkapsahv avlgyprfyk
181 lggscdagls etksainda adylnsaiak raadhgfifg dvkstfifghe icssstwihs
241 ldllnigqsy hptaagqsgg ylpvmnsva
10 //

SEQ ID No. 15

1 ccaccgccc gtcggcggc agtctcctgg cctcggctgc ggagaggfif gccgtgtgc
15 61 cgttcagcgc ggccgcgaac gtctctca ccgtgccccc gtactcgtf atcaggccct
121 tgccttctc cgaccggccc tfgaagccgg tgccttct gagcgtgacg atgtagctgc
181 ccttgatgc ggtgggggag ccggcggcga gcaccgtcc ctcggccggg gggccctggg
241 cgggcagtc ggtgaatccg cccaccgagg cgccggctgc caccggcgt atcggcggca
20 361 cggcggggt ggccgcacgg ctgtgggggg tgcgcgctc atcacgcaca cggccctgga
421 gcgtcglit ccgccctggg tfgagtaag cctcggccat ctaccgggg ggtcaaggg
481 agttgagacc ctgcatgag tctgacatga gcaccgaatc aacggggccg ttagcaccac
541 ggggcgacc ccgaaagtgc cgagaagtct tggcatggac actcctctc aacaccgta
601 gctggtacga cggttaccgc agagatcctg ctaaaaggag gttccatgag acgtccccg
25 661 ataccgcat acgtgacctc actcctctc gccgtcggct ccgccctcac cggggcagcg
721 accggcagg cgtccccagc ccggcggccc accggctatg tggccctcgg cgtactgtac
781 tgcctcggg tggcggccc cagctaccic agctccagcg gcgactgcaa gcgcagttc
841 aaggcctatc cgtacctctg gcaggcccg cattcacct cgtcgtcag ttcatggct
901 tgcctcggg cctgacggg tgatgtctg gccaatcagc tggcaccct gaactcgtc
30 961 accggcctg tctccctcac catcggaggc aacgacgcg gctctccga cgtcatgac
1021 accgtgtgc tccagtcca cagcgcctgc ctctccgca tcaacaccgc gaaggcgtac
1081 gtcgaccca cctgcccgg ccaactcagc agcgtgtaca cggcgatcag cacgaaggcc
1141 ccgtcggccc atgtggcctg cctgggctac ccccgctct acaactggg cggctcctg
1201 ctgcgggccc tctcggagac caagcggctc gccatcaacg accggcggca ctatctgaac
35 1261 agcggcatc ccaagcgcgc cggcggacc ggttcacct tggcgacgt caagagcacc
1321 ttcaccgccc atgagatctg ctccagcagc acctggctgc acagtctga cctgctgaac
1381 atggccagf cctaccacc gaccggccc ggcagctcc gggctatct gccgtcatg
1441 aacagcgtg cctgagctcc caccggcctga atfittaagg cctgaatit taaggcgaag
1501 gtaaccgga agcggaggcc ccgtcctc gggctcctg cgcacaggic accgagaacg
40 1561 gcaccgagf ggacgtctg cgcaccgggt cggcaccic gacggcgtc tctctcaga
1621 tcttccgt cgtgtctac gttgtgacga acactgct ctgctgggtc tttccggc
1681 tggccggga ggacagcgc tccagccc gatccgggac ctgcccctc tggtcacc
1741 agcggctact cacctcagc ggcaccggc ccaccgtga ggtcggcgt aactggggc
1801 cctggcgtg gggcggcgg caggcaccg agtagtggf gtcacgccc gtagccgtca
45 1861 cctcaccga ctggcggc ggggtctc taccggccc gccaccggc cctccggg
1921 tggagcccga gctgtggctg ccccgccgt cggcgtgtc gctcgggg gtttogaac
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Thermobifida\fusca\ - GDSx

5 SEQ ID No. 16

1 mgsqpraatr rrfilgipal vvtatitvl avptgretiw rrwceatqdw cigvpvdsrg
 61 qpaedgefil lspvqaatwg nyyalgdsys sgdgardyypp gtavkkgcwr sanaypelva
 10 121 eaydfaghls flacsgqrgy amidaidevg sqldwnspht slvtigiggn digfstvlkt
 181 cmvrvpilds kactdqedai rkrmakfett feelisevrt rapdanilvv gyprifpeep
 241 tgayytilas nqrwlneliq efnqqlaeav avhdeeiaas gvgvsvefvd vyhaldghei
 301 gsdepwvngv qrdlatgvt vdrstfhpna aghravgerv ieqietgpgpr plyatfavva
 361 gatvdtlage vg
 15 //

Thermobifida\fusca\ - GDSx

SEQ ID No. 17

20 1 ctgcagacac ccgccccgcc ttctcccgga tctgcatglt cggcgactcc ctacagcaca
 61 ccggcaagal gtactccaag atgcgcggct acctgccgtc ctccccgccg tactacgagg
 121 gccgctctc gaacggcccc gtcggctgg agcagctgac gaagcagltc cccggcctga
 181 cgatcgccaa cgaggccgag gggggcgcgga ccgcagtcgc ctacaacaag atctcctgga
 241 acccgaagta ccaggtcatt aacaacctcg actacgaggt caccagltc tgcagaagg
 25 301 actcgtcaa gcccgacgac ctggicatcc tgggggtggg cgccaacgac taccggcct
 361 acggttggaa cacggagcag gacgccaagc gggtcgcgga cgcctctcg gacgcggcaa
 421 accgcatggt cctgaacggc gcgaagcaga tctgctglt caacctgcc gacctgggcc
 481 agaaccggc cgcccgtccc cagaaggctc tcgaggccgt ctgcacgtg tccgctacc
 541 acaacaagct gtcctcaac ctgcccggc agctcgccc gacgggcatg gtcaagctgt
 30 601 tcgagatcga caagcagltc gcggagaigc tgcgcgaccc ccagaacttc ggctgagcg
 661 acglggagaa cccgtgtac gacggcggct acgtgtggaa gccgtcgc acccggctcc
 721 tctcgaccga ccggcagctg tggcctcti cgcgccagga gcgcctggcg atcgtggca
 781 acccgctct ggcacaggcg gtagctcgc cgatggccc cgcctggcc tgcctccta
 841 actcggaggg caagatgltc tggaccagg tccaccac caccgtggtc cagccgccc
 35 901 tctcggagcg cgcgcacc ttcacgaga ccagfacga gttcctgccc cactagicta
 961 gaggatcc

Thus, in a further aspect, the present invention provides the use of a lipolytic enzyme comprising any one of the amino acid sequences shown as SEQ ID No. 4, 5, 7, 8, 12,
 40 14, or 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, or encoded by any one of the nucleotide sequences shown as SEQ ID No. 3, 6, 9, 13, 15 or 17 or a nucleotide sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, in

a foodstuff for the preparation of a lyso-glycolipid, for example digalactosyl monoglyceride (DGMG) or monogalactosyl monoglyceride (MGMG) by treatment of a glycolipid (e.g. digalactosyl diglyceride (DGDG) or monogalactosyl diglyceride (MGDG)) with the lipolytic enzyme according to the present invention to produce the
5 partial hydrolysis product, i.e. the lyso-glycolipid.

In a further aspect, the present invention yet further provides the use of a lipolytic enzyme comprising any one of the amino acid sequences shown as SEQ ID No. 4, 5, 7, 8, 12, 14 or 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%,
10 90%, 95%, 96%, 97% or 98% identity therewith, or encoded by any one of the nucleotide sequences shown as SEQ ID No. 3, 6, 9, 13, 15 or 17 or a nucleotide sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, in a foodstuff for the preparation of a lyso-phospholipid, for example lysolecithin, by treatment of a phospholipid (e.g. lecithin) with the enzyme to
15 produce the partial hydrolysis product, i.e. a lyso-phospholipid.

In another aspect, the present invention yet further provides the use of a lipolytic enzyme comprising any one of the amino acid sequences shown as SEQ ID No. 4, 5, 7, 8, 12, 14 or 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%,
20 90%, 95%, 96%, 97% or 98% identity therewith, or encoded by any one of the nucleotide sequences shown as SEQ ID No. 3, 6, 9, 13, 15 or 17 or a nucleotide sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, in an egg or an egg-based product for the hydrolysis of phospholipids and/or glycolipids.

25

In another aspect the present invention provides the use of a lipolytic enzyme comprising any one of the amino acid sequences shown as SEQ ID No. 4, 5, 7, 8, 12, 14 or 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, or encoded by any one of the nucleotide
30 sequences shown as SEQ ID No. 3, 6, 9, 13, 15 or 17 or a nucleotide sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, in a substrate (preferably a foodstuff) for hydrolysing fatty acyl groups.

- In another aspect the present invention provides the use of a lipolytic enzyme comprising any one of the amino acid sequences shown as SEQ ID No. 4, 5, 7, 8, 12, 14 or 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, or encoded by any one of the nucleotide sequences shown as SEQ ID No. 3, 6, 9, 13, 15 or 17 or a nucleotide sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, in an edible oil for reducing the content of a phospholipid.
- 10 In a further aspect the present invention relates to the use of the lipolytic enzyme comprising any one of the amino acid sequences shown as SEQ ID No. 4, 5, 7, 8, 12, 14 or 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, or encoded by any one of the nucleotide sequences shown as SEQ ID No. 3, 6, 9, 13, 15 or 17 or a nucleotide sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, in a substrate (preferably a bioconversion mixture comprising polar lipids (preferably glycolipids)) for the production of make high value products, such as carbohydrate esters and/or protein esters and/or protein subunit esters and/or a hydroxy acid ester.
- 20 In a preferable aspect, the present invention relates to a lipolytic enzyme comprising any one of amino sequences shown as SEQ ID No. 8, 14 or 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith for the uses described herein.
- 25 More preferably the present invention relates to the use of a lipolytic enzyme comprising the amino acid sequence shown as SEQ ID No. 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.
- 30 In a broad aspect the present invention may provide a lipolytic enzyme capable of hydrolysing at least a glycolipid and/or capable of transferring an acyl group from at

least a galactolipid to one or more acyl acceptors, wherein the enzyme is obtainable, preferably obtained, from *Thermobifida* spp, preferably *T. fusca*.

5 In another broad aspect the present invention may provide a lipolytic enzyme capable of hydrolysing at least a glycolipid and/or capable of transferring an acyl group from at least a galactolipid to one or more acyl acceptors, wherein the enzyme is obtainable, preferably obtained, from *Corynebacterium* spp, preferably *C. efficiens*.

10 In another broad aspect the present invention may provide a lipolytic enzyme capable of hydrolysing at least a glycolipid and/or capable of transferring an acyl group from at least a galactolipid to one or more acyl acceptors, wherein the enzyme is obtainable, preferably obtained, from *Streptomyces avermitilis*.

15 In a further aspect the present invention may provide a lipolytic enzyme capable of hydrolysing at least a glycolipid and/or capable of transferring an acyl group from at least a galactolipid to one or more acyl acceptors, wherein the enzyme comprises SEQ ID No. 5, 7, 8, 12, or 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, or the enzyme is encoded by any one of the nucleotide sequences shown as SEQ ID No. 6, 9, 13, or 17 or a
20 nucleotide sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

25 In a further aspect the present invention may provide a lipolytic enzyme capable of hydrolysing at least a glycolipid and/or capable of transferring an acyl group from at least a galactolipid to one or more acyl acceptors, wherein the enzyme comprises SEQ ID No. 14 or an amino acid sequence which has at least 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, or the enzyme is encoded by any one of the nucleotide sequences shown as SEQ ID No. 15 or a nucleotide sequence which has at least 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

30

In a further aspect the present invention may provide a lipolytic enzyme capable of hydrolysing at least a glycolipid and/or capable of transferring an acyl group from at

least a galactolipid to one or more acyl acceptors, wherein the enzyme comprises SEQ ID No. 16 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, or the enzyme is encoded by any one of the nucleotide sequences shown as SEQ ID No. 17 or a nucleotide sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

In one embodiment of the present invention preferably the *Streptomyces* species from which the lipolytic enzyme is obtainable (or obtained) is not *Streptomyces rimosus*.

10 In one embodiment of the present invention preferably the *Streptomyces* species from which the lipolytic enzyme is obtainable (or obtained) is not *Streptomyces coelicolor*.

ADVANTAGES

15 One advantage of the present invention is that the lipolytic enzyme has significant glycolipid hydrolysing activity. This was surprising for a lipolytic enzyme from *Streptomyces* spp. In addition, this was surprising for a lipolytic enzyme from *Thermobifida* and *Corynebacterium* spp.

20 A further advantage of the present invention is that the lipolytic enzyme has no or no significant triacylglycerol hydrolysing activity.

ISOLATED

25 In one aspect, preferably the sequence is in an isolated form. The term "isolated" means that the sequence is at least substantially free from at least one other component with which the sequence is naturally associated in nature and as found in nature.

PURIFIED

In one aspect, preferably the sequence is in a purified form. The term "purified" means that the sequence is in a relatively pure state – e.g. at least about 90% pure, or at least about 95% pure or at least about 98% pure.

NUCLEOTIDE SEQUENCE

The scope of the present invention encompasses nucleotide sequences encoding enzymes having the specific properties as defined herein.

The term "nucleotide sequence" as used herein refers to an oligonucleotide sequence or polynucleotide sequence, and variants, homologues, fragments and derivatives thereof (such as portions thereof). The nucleotide sequence may be of genomic or synthetic or recombinant origin, which may be double-stranded or single-stranded whether representing the sense or anti-sense strand.

The term "nucleotide sequence" in relation to the present invention includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA sequence coding for the present invention.

In a preferred embodiment, the nucleotide sequence when relating to and when encompassed by the *per se* scope of the present invention does not include the native nucleotide sequence according to the present invention when in its natural environment and when it is linked to its naturally associated sequence(s) that is/are also in its/their natural environment. For ease of reference, we shall call this preferred embodiment the "non-native nucleotide sequence". In this regard, the term "native nucleotide sequence" means an entire nucleotide sequence that is in its native environment and when operatively linked to an entire promoter with which it is naturally associated, which promoter is also in its native environment. However, the amino acid sequence encompassed by scope the present invention can be isolated and/or purified post expression of a nucleotide sequence in its native organism. Preferably, however, the

amino acid sequence encompassed by scope of the present invention may be expressed by a nucleotide sequence in its native organism but wherein the nucleotide sequence is not under the control of the promoter with which it is naturally associated within that organism.

5

PREPARATION OF THE NUCLEOTIDE SEQUENCE

Typically, the nucleotide sequence encompassed by scope of the present invention is prepared using recombinant DNA techniques (i.e. recombinant DNA). However, in an
10 alternative embodiment of the invention, the nucleotide sequence could be synthesised, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al.*, (1980) *Nuc Acids Res Symp Ser* 215-23 and Horn T *et al.*, (1980) *Nuc Acids Res Symp Ser* 225-232).

15 A nucleotide sequence encoding an enzyme which has the specific properties as defined herein may be identified and/or isolated and/or purified from any cell or organism producing said enzyme. Various methods are well known within the art for the identification and/or isolation and/or purification of nucleotide sequences. By way of example, PCR amplification techniques to prepare more of a sequence may be used
20 once a suitable sequence has been identified and/or isolated and/or purified.

By way of further example, a genomic DNA and/or cDNA library may be constructed using chromosomal DNA or messenger RNA from the organism producing the enzyme. If the amino acid sequence of the enzyme or a part of the amino acid
25 sequence of the enzyme is known, labelled oligonucleotide probes may be synthesised and used to identify enzyme-encoding clones from the genomic library prepared from the organism. Alternatively, a labelled oligonucleotide probe containing sequences homologous to another known enzyme gene could be used to identify enzyme-encoding clones. In the latter case, hybridisation and washing conditions of lower
30 stringency are used.

Alternatively, enzyme-encoding clones could be identified by inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar plates containing a substrate for the enzyme (e.g. maltose for a glucosidase (maltase) producing enzyme), thereby allowing clones
5 expressing the enzyme to be identified.

In a yet further alternative, the nucleotide sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite
10 method described by Beucage S.L. *et al.*, (1981) *Tetrahedron Letters* 22, p 1859-1869, or the method described by Matthes *et al.*, (1984) *EMBO J.* 3, p 801-805. In the phosphoroamidite method, oligonucleotides are synthesised, e.g. in an automatic DNA synthesiser, purified, annealed, ligated and cloned in appropriate vectors.

15 The nucleotide sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin, or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate) in accordance with standard techniques. Each ligated fragment corresponds to various parts of the entire nucleotide sequence. The DNA sequence may also be prepared by polymerase chain
20 reaction (PCR) using specific primers, for instance as described in US 4,683,202 or in Saiki R K *et al.*, (*Science* (1988) 239, pp 487-491).

Due to degeneracy in the genetic code, nucleotide sequences may be readily produced in which the triplet codon usage, for some or all of the amino acids encoded by the
25 original nucleotide sequence, has been changed thereby producing a nucleotide sequence with low homology to the original nucleotide sequence but which encodes the same, or a variant, amino acid sequence as encoded by the original nucleotide sequence. For example, for most amino acids the degeneracy of the genetic code is at the third position in the triplet codon (wobble position) (for reference see Stryer,
30 Lubert, *Biochemistry*, Third Edition, Freeman Press, ISBN 0-7167-1920-7) therefore, a nucleotide sequence in which all triplet codons have been "wobbled" in the third position would be about 66% identical to the original nucleotide sequence however,

the amended nucleotide sequence would encode for the same, or a variant, primary amino acid sequence as the original nucleotide sequence.

Therefore, the present invention further relates to any nucleotide sequence that has
5 alternative triplet codon usage for at least one amino acid encoding triplet codon, but which encodes the same, or a variant, polypeptide sequence as the polypeptide sequence encoded by the original nucleotide sequence.

Furthermore, specific organisms typically have a bias as to which triplet codons are
10 used to encode amino acids. Preferred codon usage tables are widely available, and can be used to prepare codon optimised genes. Such codon optimisation techniques are routinely used to optimise expression of transgenes in a heterologous host.

MOLECULAR EVOLUTION

15 Once an enzyme-encoding nucleotide sequence has been isolated, or a putative enzyme-encoding nucleotide sequence has been identified, it may be desirable to modify the selected nucleotide sequence, for example it may be desirable to mutate the sequence in order to prepare an enzyme in accordance with the present invention.

20 Mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites.

A suitable method is disclosed in Morinaga *et al* (Biotechnology (1984) 2, p646-649).
Another method of introducing mutations into enzyme-encoding nucleotide sequences
25 is described in Nelson and Long (Analytical Biochemistry (1989), 180, p 147-151).

Instead of site directed mutagenesis, such as described above, one can introduce mutations randomly for instance using a commercial kit such as the GeneMorph PCR mutagenesis kit from Stratagene, or the Diversify PCR random mutagenesis kit from
30 Clontech. EP 0 583 265 refers to methods of optimising PCR based mutagenesis, which can also be combined with the use of mutagenic DNA analogues such as those

described in EP 0 866 796. Error prone PCR technologies are suitable for the production of variants of lipolytic enzymes with preferred characteristics. WO0206457 refers to molecular evolution of lipases.

5 A third method to obtain novel sequences is to fragment non-identical nucleotide sequences, either by using any number of restriction enzymes or an enzyme such as Dnase I, and reassembling full nucleotide sequences coding for functional proteins. Alternatively one can use one or multiple non-identical nucleotide sequences and introduce mutations during the reassembly of the full nucleotide sequence. DNA
10 shuffling and family shuffling technologies are suitable for the production of variants of lipid acyl transferases with preferred characteristics. Suitable methods for performing 'shuffling' can be found in EP0 752 008, EP1 138 763, EP1 103 606. Shuffling can also be combined with other forms of DNA mutagenesis as described in US 6,180,406 and WO 01/34835.

15

Thus, it is possible to produce numerous site directed or random mutations into a nucleotide sequence, either *in vivo* or *in vitro*, and to subsequently screen for improved functionality of the encoded polypeptide by various means. Using *in silico* and *ex* mediated recombination methods (see WO 00/58517, US 6,344,328, US 6,361,974),
20 for example, molecular evolution can be performed where the variant produced retains very low homology to known enzymes or proteins. Such variants thereby obtained may have significant structural analogy to known lipolytic enzymes, but have very low amino acid sequence homology.

25 As a non-limiting example, In addition, mutations or natural variants of a polynucleotide sequence can be recombined with either the wild type or other mutations or natural variants to produce new variants. Such new variants can also be screened for improved functionality of the encoded polypeptide.

30 The application of the above-mentioned and similar molecular evolution methods allows the identification and selection of variants of the enzymes of the present invention which have preferred characteristics without any prior knowledge of protein

structure or function, and allows the production of non-predictable but beneficial mutations or variants. There are numerous examples of the application of molecular evolution in the art for the optimisation or alteration of enzyme activity, such examples include, but are not limited to one or more of the following: optimised expression and/or activity in a host cell or in vitro, increased enzymatic activity, altered substrate and/or product specificity, increased or decreased enzymatic or structural stability, altered enzymatic activity/specificity in preferred environmental conditions, e.g. temperature, pH, substrate

5 As will be apparent to a person skilled in the art, using molecular evolution tools an enzyme may be altered to improve the functionality of the enzyme.

Suitably, the lipolytic enzyme used in the invention may be a variant, i.e. may contain at least one amino acid substitution, deletion or addition, when compared to a parental enzyme. Variant enzymes retain at least 20%, 30%, 40%, 50 %, 60%, 70%, 80%, 90%, 15 95%, 97%, 99% homology with the parent enzyme. Suitable parent enzymes may include any enzyme with esterase or lipase activity. Preferably, the parent enzyme aligns to the pfam00657 consensus sequence.

20 In a preferable embodiment a variant lipolytic enzyme retains or incorporates at least one or more of the pfam00657 consensus sequence amino acid residues found in the GDSx, GANDY and HPT blocks.

Enzymes, such as lipases with no or low galactolipase and/or phospholipase activity in 25 an aqueous environment may be mutated using molecular evolution tools to introduce or enhance the galactolipase and/or phospholipase activity, thereby producing a lipolytic enzyme with significant galactolipase and/or phospholipase activity suitable for use in the compositions and methods of the present invention.

30 Suitably the variant enzyme may have no activity on triglycerides and/or monoglycerides and/or diglycerides.

Alternatively, the variant enzyme for use in the invention may have increased activity on triglycerides, and/or may also have increased activity on one or more of the following, polar lipids, phospholipids, lecithin, phosphatidylcholine, glycolipids, digalactosyl monoglyceride, monogalactosyl monoglyceride.

5

AMINO ACID SEQUENCES

The scope of the present invention also encompasses amino acid sequences of enzymes having the specific properties as defined herein.

10

As used herein, the term "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide". In some instances, the term "amino acid sequence" is synonymous with the term "enzyme".

15

The amino acid sequence may be prepared/isolated from a suitable source, or it may be made synthetically or it may be prepared by use of recombinant DNA techniques.

The enzyme encompassed in the present invention may be used in conjunction with other enzymes. Thus the present invention also covers a combination of enzymes wherein the combination comprises the enzyme of the present invention and another enzyme, which may be another enzyme according to the present invention. This aspect is discussed in a later section.

25 Preferably the amino acid sequence when relating to and when encompassed by the *per se* scope of the present invention is not a native enzyme. In this regard, the term "native enzyme" means an entire enzyme that is in its native environment and when it has been expressed by its native nucleotide sequence.

30

IDENTITY/HOMOLOGY

The present invention also encompasses the use of homologues of any amino acid sequence of an enzyme or of any nucleotide sequence encoding such an enzyme.

5

Here, the term "homologue" means an entity having a certain homology with the amino acid sequences and the nucleotide sequences. Here, the term "homology" can be equated with "identity". These terms will be used interchangeably herein.

10 In the present context, a homologous amino acid sequence is taken to include an amino acid sequence which may be at least 87 or 90% identical, preferably at least 95, 96, 97, 98 or 99% identical to the sequence. Typically, the homologues will comprise the same active sites etc. – e.g. as the subject amino acid sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar
15 chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

In the present context, an homologous nucleotide sequence is taken to include a nucleotide sequence which may be at least 85 or 90% identical, preferably at least 95,
20 96, 97, 98 or 99% identical to a nucleotide sequence encoding an enzyme of the present invention (the subject sequence). Typically, the homologues will comprise the same sequences that code for the active sites etc. as the subject sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it
25 is preferred to express homology in terms of sequence identity.

For the amino acid sequences and the nucleotide sequences, homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate
30 % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux *et al* 1984 Nuc. Acids Research 12 p387). Examples of other software than

can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *Short Protocols in Molecular Biology*, 4th Ed – Chapter 18), FASTA (Altschul *et al.*, 1990 *J. Mol. Biol.* 403-410) and the GENWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online
5 searching (see Ausubel *et al.*, 1999, *Short Protocols in Molecular Biology*, pages 7-58 to 7-60).

However, for some applications, it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide
10 sequence (see *FEMS Microbiol Lett* 1999 174(2): 247-50; and *FEMS Microbiol Lett* 1999 177(1): 187-8).

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a
15 scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for
20 further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Alternatively, percentage homologies may be calculated using the multiple alignment
25 feature in DNASIS™ (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), *Gene* 73(1), 237-244).

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of
30 the sequence comparison and generates a numerical result.

In a preferable aspect of the present invention the following software and settings for calculating percentage sequence homology/identity are used. For amino acid sequences percentage of identities (homology) or "positives" are calculated by the AlignX VectorNTI (Vector NTI Advance 9.1 from Invitrogen Corporation, Carlsbad, California, USA.) for each possible pair of amino acid sequences. Settings are default parameters (Gap opening penalty - 10, Gap extension penalty 0.1).

For nucleic acid sequences percentage of identities (homology) or "positives" are calculated by the AlignX VectorNTI programme from Informax Inc. (USA) for each possible pair of nucleic acid sequences. Settings are default settings which for DNA is: Gap opening penalty: 15 and Gap extension penalty: 6.66. (same settings for multiple alignments).

Preferably the amino acid identity (homology) is calculated across the full-length amino acid sequence (e.g. SEQ IDs 4, 5, 7, 8, 10, 12 and 14), or for nucleic acid to a corresponding polynucleotide which encodes the respective the full-length amino acid sequence. Amino acid or nucleic acid identity (homology) may be, preferably, calculated by comparing the homology/identity over the mature polypeptide sequence, i.e. a polypeptide sequence which has been co- or post-translationally processed, for example by cleavage of an N-terminal signal peptide, or a C-terminal cleavage event.

The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in amino acid properties (such as polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues) and it is therefore useful to group amino acids together in functional groups. Amino acids can be grouped together based on the properties of their side chain alone. However it is more useful to include mutation data as well. The sets of amino acids thus derived are likely to be conserved for structural reasons. These sets can be described in the form of a Venn diagram (Livingstone C.D. and Barton G.J. (1993) "Protein sequence alignments: a strategy for the hierarchical analysis of residue conservation" *Comput. Appl Biosci.* 9:

745-756)(Taylor W.R. (1986) "The classification of amino acid conservation" *J.Theor.Biol.* 119; 205-218). Conservative substitutions may be made, for example according to the table below which describes a generally accepted Venn diagram grouping of amino acids.

5

| SET | | SUB-SET | |
|-------------|-------------------------|--------------------|-----------|
| Hydrophobic | F W Y H K M I L V A G C | Aromatic | F W Y H |
| | | Aliphatic | I L V |
| Polar | W Y H K R E D C S T N Q | Charged | H K R E D |
| | | Positively charged | H K R |
| | | Negatively charged | E D |
| Small | V C A G S P T N D | Tiny | A G S |

The present invention also encompasses homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) that may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyrilylalanine, thienylalanine, naphthylalanine and phenylglycine.

Replacements may also be made by unnatural amino acids.

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β -alanine residues. A further form of variation involves the presence of one or more

amino acid residues in peptoid form, and will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α -carbon substituent group is on the residue's nitrogen atom rather than the α -carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ *et al.*, *PNAS* (1992) 89(20), 9367-9371 and Horwell DC, *Trends Biotechnol.* (1995) 13(4), 132-134.

The nucleotide sequences for use in the present invention may include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones and/or the addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of nucleotide sequences of the present invention.

The present invention also encompasses the use of nucleotide sequences that are complementary to the sequences presented herein, or any derivative, fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence can be used as a probe to identify similar coding sequences in other organisms etc.

Polynucleotides which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. In addition, other homologues may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other species, and probing such libraries with probes comprising all or part of any one of the sequences in the attached sequence listings under conditions of medium to high stringency. Similar considerations

apply to obtaining species homologues and allelic variants of the polypeptide or nucleotide sequences of the invention.

Variants and strain/species homologues may also be obtained using degenerate PCR
5 which will use primers designed to target sequences within the variants and homologues
encoding conserved amino acid sequences within the sequences of the present invention.
Conserved sequences can be predicted, for example, by aligning the amino acid
sequences from several variants/homologues. Sequence alignments can be performed
using computer software known in the art. For example the GCG Wisconsin PileUp
10 program is widely used.

The primers used in degenerate PCR will contain one or more degenerate positions and
will be used at stringency conditions lower than those used for cloning sequences with
single sequence primers against known sequences.

15 Alternatively, such polynucleotides may be obtained by site directed mutagenesis of
characterised sequences. This may be useful where for example silent codon sequence
changes are required to optimise codon preferences for a particular host cell in which the
polynucleotide sequences are being expressed. Other sequence changes may be desired
20 in order to introduce restriction enzyme recognition sites, or to alter the property or
function of the polypeptides encoded by the polynucleotides.

Polynucleotides (nucleotide sequences) of the invention may be used to produce a primer,
e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled
25 with a revealing label by conventional means using radioactive or non-radioactive labels,
or the polynucleotides may be cloned into vectors. Such primers, probes and other
fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40
nucleotides in length, and are also encompassed by the term polynucleotides of the
invention as used herein.

30

Polynucleotides such as DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

- 5 In general, primers will be produced by synthetic means, involving a stepwise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

10 Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

BIOLOGICALLY ACTIVE

15

Preferably, the variant sequences etc. are at least as biologically active as the sequences presented herein.

20 As used herein "biologically active" refers to a sequence having a similar structural function (but not necessarily to the same degree), and/or similar regulatory function (but not necessarily to the same degree), and/or similar biochemical function (but not necessarily to the same degree) of the naturally occurring sequence.

HYBRIDISATION

25

The present invention also encompasses sequences that are complementary to the nucleic acid sequences of the present invention or sequences that are capable of hybridising either to the sequences of the present invention or to sequences that are complementary thereto.

30

The term "hybridisation" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies.

5

The present invention also encompasses the use of nucleotide sequences that are capable of hybridising to the sequences that are complementary to the sequences presented herein, or any derivative, fragment or derivative thereof.

10 The term "variant" also encompasses sequences that are complementary to sequences that are capable of hybridising to the nucleotide sequences presented herein.

Preferably, the term "variant" encompasses sequences that are complementary to sequences that are capable of hybridising under stringent conditions (e.g. 50°C and
15 0.2xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na₃citrate pH 7.0}) to the nucleotide sequences presented herein.

More preferably, the term "variant" encompasses sequences that are complementary to sequences that are capable of hybridising under high stringent conditions (e.g. 65°C
20 and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na₃citrate pH 7.0}) to the nucleotide sequences presented herein.

The present invention also relates to nucleotide sequences that can hybridise to the nucleotide sequences of the present invention (including complementary sequences of
25 those presented herein).

The present invention also relates to nucleotide sequences that are complementary to sequences that can hybridise to the nucleotide sequences of the present invention (including complementary sequences of those presented herein).

30

Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridising to the nucleotide sequences presented herein under conditions of intermediate to maximal stringency.

- 5 In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention, or the complement thereof, under stringent conditions (e.g. 50°C and 0.2xSSC).

- In a more preferred aspect, the present invention covers nucleotide sequences that can
10 hybridise to the nucleotide sequence of the present invention, or the complement thereof, under high stringent conditions (e.g. 65°C and 0.1xSSC).

RECOMBINANT

- 15 In one aspect the sequence for use in the present invention is a recombinant sequence – i.e. a sequence that has been prepared using recombinant DNA techniques.

- These recombinant DNA techniques are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature, for example, J. Sambrook,
20 E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press.

SYNTHETIC

- 25 In one aspect the sequence for use in the present invention is a synthetic sequence – i.e. a sequence that has been prepared by *in vitro* chemical or enzymatic synthesis. It includes, but is not limited to, sequences made with optimal codon usage for host organisms - such as the methylotrophic yeasts *Pichia* and *Hansenula*.

EXPRESSION OF ENZYMES

The nucleotide sequence for use in the present invention may be incorporated into a recombinant replicable vector. The vector may be used to replicate and express the nucleotide sequence, in enzyme form, in and/or from a compatible host cell.

Expression may be controlled using control sequences e.g. regulatory sequences.

The enzyme produced by a host recombinant cell by expression of the nucleotide sequence may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. The coding sequences may be designed with signal sequences which direct secretion of the substance coding sequences through a particular prokaryotic or eukaryotic cell membrane.

EXPRESSION VECTOR

The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

Preferably, the expression vector is incorporated into the genome of a suitable host organism. The term "incorporated" preferably covers stable incorporation into the genome.

The nucleotide sequence of the present invention may be present in a vector in which the nucleotide sequence is operably linked to regulatory sequences capable of providing for the expression of the nucleotide sequence by a suitable host organism.

The vectors for use in the present invention may be transformed into a suitable host cell as described below to provide for expression of a polypeptide of the present invention.

30

The choice of vector e.g. a plasmid, cosmid, or phage vector will often depend on the host cell into which it is to be introduced.

The vectors for use in the present invention may contain one or more selectable marker genes- such as a gene, which confers antibiotic resistance e.g. ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Alternatively, the selection may be
5 accomplished by co-transformation (as described in WO91/17243).

Vectors may be used *in vitro*, for example for the production of RNA or used to transfect, transform, transduce or infect a host cell.

10 Thus, in a further embodiment, the invention provides a method of making nucleotide sequences of the present invention by introducing a nucleotide sequence of the present invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector.

15 The vector may further comprise a nucleotide sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

REGULATORY SEQUENCES

20

In some applications, the nucleotide sequence for use in the present invention is operably linked to a regulatory sequence which is capable of providing for the expression of the nucleotide sequence, such as by the chosen host cell. By way of example, the present invention covers a vector comprising the nucleotide sequence of
25 the present invention operably linked to such a regulatory sequence, i.e. the vector is an expression vector.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A
30 regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals.

- 5 The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site.

Enhanced expression of the nucleotide sequence encoding the enzyme of the present invention may also be achieved by the selection of heterologous regulatory regions,
10 e.g. promoter, secretion leader and terminator regions.

Preferably, the nucleotide sequence according to the present invention is operably linked to at least a promoter.

- 15 Examples of suitable promoters for directing the transcription of the nucleotide sequence in a bacterial, fungal or yeast host are well known in the art.

CONSTRUCTS

- 20 The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes a nucleotide sequence for use according to the present invention directly or indirectly attached to a promoter.

An example of an indirect attachment is the provision of a suitable spacer group such as
25 an intron sequence, such as the Sh1-intron or the ADH intron, intermediate the promoter and the nucleotide sequence of the present invention. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. In some cases, the terms do not cover the natural combination of the nucleotide sequence coding for the protein ordinarily associated with the wild type gene promoter and when
30 they are both in their natural environment.

The construct may even contain or express a marker, which allows for the selection of the genetic construct.

For some applications, preferably the construct of the present invention comprises at least
5 the nucleotide sequence of the present invention operably linked to a promoter.

HOST CELLS

10 The term "host cell" - in relation to the present invention includes any cell that comprises either the nucleotide sequence or an expression vector as described above and which is used in the recombinant production of an enzyme having the specific properties as defined herein.

15 Thus, a further embodiment of the present invention provides host cells transformed or transfected with a nucleotide sequence that expresses the enzyme of the present invention. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial), fungal, yeast or plant cells. Preferably, the host cells are not human cells.

20

Examples of suitable bacterial host organisms are gram positive or gram negative bacterial species.

Depending on the nature of the nucleotide sequence encoding the enzyme of the
25 present invention, and/or the desirability for further processing of the expressed protein, eukaryotic hosts such as yeasts or other fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from the yeast cell, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these instances, a
30 different fungal host organism should be selected.

The use of suitable host cells - such as yeast, fungal and plant host cells - may provide for post-translational modifications (e.g. myristoylation, glycosylation, truncation, lipidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the present invention.

The host cell may be a protease deficient or protease minus strain.

The genotype of the host cell may be modified to improve expression.

Examples of host cell modifications include protease deficiency, supplementation of rare tRNA's, and modification of the reductive potential in the cytoplasm to enhance disulphide bond formation.

For example, the host cell *E. coli* may overexpress rare tRNA's to improve expression of heterologous proteins as exemplified/described in Kane (*Curr Opin Biotechnol* (1995), 6, 494-500 "Effects of rare codon clusters on high-level expression of heterologous proteins in *E.coli*"). The host cell may be deficient in a number of reducing enzymes thus favouring formation of stable disulphide bonds as exemplified/described in Bessette (*Proc Natl Acad Sci USA* (1999), 96, 13703-13708 "Efficient folding of proteins with multiple disulphide bonds in the *Escherichia coli* cytoplasm").

In one embodiment the host cell is a bacteria, preferably a gram-positive bacteria, preferably a host cell selected from *Actinobacteria*, such as *Bifidobacteria* and *Aeromonas*, particularly preferably *Aeromonas salmonicida*. Still more preferred are Actinomycetales such as *Corynebacteria*, in particular *Corynebacterium glutamicum* and *Nocardia*. Particularly preferred are *Streptomyetaceae*, such as *Streptomyces*, especially *S. lividans*.

A microbial host can be used for expression of the galactolipase gene, e.g. *Eubacteria*, *Archea* or *Fungi*, including yeast. Preferred are *Eubacteria*, for example, *Firmicutes*

(low GC-Gram positive bacteria), such as *Bacillus subtilis* and other *bacillus* species, lactic acid bacteria such as species of genera *Lactobacillus* and *Lactococcus*.

Also preferred are Gram-negative *Proteobacteria*, in particular *Gammaproteobacteria*,
5 such as host species belonging to the genera *Pseudomonas*, *Xanthomonas*, *Citrobacter*
and *Escherichia*, especially *Escherichia coli*.

In another embodiment the host cell is the same genus as the native host species, i.e.
the recombinant gene is re-introduced and expressed in a species from the same genus
10 as the species from which the recombinant gene was isolated.

In another embodiment the host cell is the native host species, i.e. the recombinant
gene is re-introduced and expressed in the same species from which the recombinant
gene was isolated.

15

ORGANISM

The term "organism" in relation to the present invention includes any organism that could
comprise the nucleotide sequence coding for the enzyme according to the present
20 invention and/or products obtained therefrom, and/or wherein a promoter can allow
expression of the nucleotide sequence according to the present invention when present in
the organism.

Suitable organisms may include a prokaryote, fungus, yeast or a plant.

25

The term "transgenic organism" in relation to the present invention includes any organism
that comprises the nucleotide sequence coding for the enzyme according to the present
invention and/or the products obtained therefrom, and/or wherein a promoter can allow
expression of the nucleotide sequence according to the present invention within the
30 organism. Preferably the nucleotide sequence is incorporated in the genome of the
organism.

The term "transgenic organism" does not cover native nucleotide coding sequences in their natural environment when they are under the control of their native promoter which is also in its natural environment.

5 Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, the nucleotide sequence coding for the enzyme according to the present invention, constructs according to the present invention, vectors according to the present invention, plasmids according to the present invention, cells according to the present invention, tissues according to the present invention, or the
10 products thereof.

For example the transgenic organism may also comprise the nucleotide sequence coding for the enzyme of the present invention under the control of a heterologous promoter.

15 TRANSFORMATION OF HOST CELLS/ORGANISM

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*.

20 Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook *et al* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press). If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as by removal of introns.

25 Filamentous fungi cells may be transformed using various methods known in the art - such as a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known. The use of *Aspergillus* as a host microorganism is described in EP 0 238 023.

30 Another host organism can be a plant. A review of the general techniques used for transforming plants may be found in articles by Potrykus (*Annu Rev Plant Physiol*

Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). Further teachings on plant transformation may be found in EP-A-0449375.

- 5 General teachings on the transformation of fungi, yeasts and plants are presented in following sections.

TRANSFORMED FUNGUS

- 10 A host organism may be a fungus - such as a filamentous fungus. Examples of suitable such hosts include any member belonging to the genera *Thermomyces*, *Acremonium*, *Aspergillus*, *Penicillium*, *Mucor*, *Neurospora*, *Trichoderma* and the like.

- 15 Teachings on transforming filamentous fungi are reviewed in US-A-5741665 which states that standard techniques for transformation of filamentous fungi and culturing the fungi are well known in the art. An extensive review of techniques as applied to *N. crassa* is found, for example in Davis and de Serres, *Methods Enzymol* (1971) 17A: 79-143.

- 20 Further teachings on transforming filamentous fungi are reviewed in US-A-5674707.

In one aspect, the host organism can be of the genus *Aspergillus*, such as *Aspergillus niger*.

- 25 A transgenic *Aspergillus* according to the present invention can also be prepared by following, for example, the teachings of Turner G. 1994 (Vectors for genetic manipulation. In: Martinelli S.D., Kinghorn J.R.(Editors) *Aspergillus: 50 years on. Progress in industrial microbiology* vol 29. Elsevier Amsterdam 1994. pp. 641-666).

Gene expression in filamentous fungi has been reviewed in Punt *et al.* (2002) Trends Biotechnol 2002 May;20(5):200-6, Archer & Peberdy Crit Rev Biotechnol (1997) 17(4):273-306.

5

TRANSFORMED YEAST

In another embodiment, the transgenic organism can be a yeast.

- 10 A review of the principles of heterologous gene expression in yeast are provided in, for example, *Methods Mol Biol* (1995), 49:341-54, and *Curr Opin Biotechnol* (1997) Oct;8(5):554-60

- 15 In this regard, yeast – such as the species *Saccharomyces cerevisiae* or *Pichia pastoris* (see FEMS Microbiol Rev (2000 24(1):45-66), may be used as a vehicle for heterologous gene expression.

- 20 A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", *Yeasts*, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

- 25 For the transformation of yeast, several transformation protocols have been developed. For example, a transgenic *Saccharomyces* according to the present invention can be prepared by following the teachings of Hinnen *et al.*, (1978, *Proceedings of the National Academy of Sciences of the USA* 75, 1929); Beggs, J D (1978, *Nature*, London, 275, 104); and Ito, H *et al* (1983, *J Bacteriology* 153, 163-168).

- 30 The transformed yeast cells may be selected using various selective markers – such as auxotrophic markers dominant antibiotic resistance markers.

TRANSFORMED PLANTS/PLANT CELLS

A host organism suitable for the present invention may be a plant. A review of the general techniques may be found in articles by Potrykus (*Annu Rev Plant Physiol Plant Mol Biol* [1991] 42:205-225) and Christou (*Agro-Food-Industry Hi-Tech* March/April 1994 17-27).

CULTURING AND PRODUCTION

Host cells transformed with the nucleotide sequence of the present invention may be cultured under conditions conducive to the production of the encoded enzyme and which facilitate recovery of the enzyme from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in questions and obtaining expression of the enzyme.

The protein produced by a recombinant cell may be displayed on the surface of the cell.

The enzyme may be secreted from the host cells and may conveniently be recovered from the culture medium using well-known procedures.

SECRETION

Often, it is desirable for the enzyme to be secreted from the expression host into the culture medium from where the enzyme may be more easily recovered. According to the present invention, the secretion leader sequence may be selected on the basis of the desired expression host. Hybrid signal sequences may also be used with the context of the present invention.

Typical examples of heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene (*glaA* - both 18 and 24 amino acid

versions e.g. from *Aspergillus*), the a-factor gene (yeasts e.g. *Saccharomyces*, *Kluyveromyces* and *Hansenula*) or the α -amylase gene (*Bacillus*).

By way of example, the secretion of heterologous proteins in *E. coli* is reviewed in
5 Methods Enzymol (1990) 182:132-43.

DETECTION

A variety of protocols for detecting and measuring the expression of the amino acid
10 sequence are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS).

A wide variety of labels and conjugation techniques are known by those skilled in the
15 art and can be used in various nucleic and amino acid assays.

A number of companies such as Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits and protocols for these procedures.

20 Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US-A-3,817,837; US-A-3,850,752; US-A-3,939,350; US-A-3,996,345; US-A-
25 4,277,437; US-A-4,275,149 and US-A-4,366,241.

Also, recombinant immunoglobulins may be produced as shown in US-A-4,816,567.

30 FUSION PROTEINS

The amino acid sequence for use according to the present invention may be produced as a fusion protein, for example to aid in extraction and purification. Examples of

fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and (β -galactosidase). It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences.

5

Preferably, the fusion protein will not hinder the activity of the protein sequence.

Gene fusion expression systems in *E. coli* have been reviewed in *Curr Opin Biotechnol* (1995) 6(5):501-6.

10

In another embodiment of the invention, the amino acid sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for agents capable of affecting the substance activity, it may be useful to encode a chimeric substance expressing a heterologous epitope that is recognised by a commercially available antibody.

15

LARGE SCALE APPLICATION

In one preferred embodiment of the present invention, the amino acid sequence is used for large scale applications.

20

Preferably the amino acid sequence is produced in a quantity of from 1g per litre to about 2g per litre of the total cell culture volume after cultivation of the host organism.

Preferably the amino acid sequence is produced in a quantity of from 100mg per litre to about 900mg per litre of the total cell culture volume after cultivation of the host organism.

25

Preferably the amino acid sequence is produced in a quantity of from 250mg per litre to about 500mg per litre of the total cell culture volume after cultivation of the host organism.

30

FOOD

The composition of the present invention may be used as – or in the preparation of - a
5 food. Here, the term “food” is used in a broad sense – and covers food for humans as
well as food for animals (i.e. a feed). In a preferred aspect, the food is for human
consumption.

The food may be in the form of a solution or as a solid – depending on the use and/or
10 the mode of application and/or the mode of administration.

FOOD INGREDIENT

The composition of the present invention may be used as a food ingredient.
15

As used herein the term “food ingredient” includes a formulation, which is or can be
added to functional foods or foodstuffs and includes formulations which can be used at
low levels in a wide variety of products that require, for example, acidifying or
emulsifying.

20 The food ingredient may be in the form of a solution or as a solid – depending on the
use and/or the mode of application and/or the mode of administration.

FOOD PRODUCTS

25 The composition of the present invention can be used in the preparation of food
products such as one or more of: confectionery products, dairy products, meat
products, poultry products, fish products and bakery products.

30 The present invention also provides a method of preparing a food or a food ingredient, the
method comprising admixing a lipolytic enzyme according to the present invention with
another food ingredient.

Further preferable aspects are presented in the accompanying claims and in the following Figures and examples.

5 Figure 1 shows PCR fragment SEQ ID No. 1, which is a partial non-enzyme encoding polynucleotide; this sequence is a ribosomal 16S RNA gene widely used for taxonomic comparisons;

10 Figure 2 shows PCR fragment SEQ ID No. 2, which is a partial non-enzyme encoding polynucleotide; this sequence is a ribosomal 16S RNA gene widely used for taxonomic comparisons;

Figure 3 shows a polynucleotide encoding a lipolytic enzyme according to the present invention (SEQ ID No. 3);

15

Figure 4 shows an amino acid sequence of a lipolytic enzyme according to the present invention (SEQ ID No. 4);

20 Figure 5 shows the structure of the lipolytic enzyme expression vectors pGTK(L131) and pET11(131-51);

Figure 6 shows a graph of the effect of a lipolytic enzyme from *Streptomyces sp.* L130 on digalactosyldiglyceride in dough;

25 Figure 7 shows in graphical form the effect of a lipolytic enzyme from *Streptomyces sp.* L131 on digalactosyldiglyceride in dough;

Figure 8 shows in graphical form the effect of a lipolytic enzyme from *Streptomyces* on triglyceride in dough;

30

Figure 9 shows the pH profile of the lipolytic enzyme obtained from *Streptomyces sp.* L131 on galactolipid substrate;

Figure 10 shows a TLC plate of lipids extracted from dough treated with a lipolytic enzyme from *Streptomyces* expressed in *E.coli* labelled #236; Lane 1=control; Lane2=#236, 0.225PLU-7/g flour; Lane 3=#236, 0.45 PLU-7/g flour; Lane 4=#236, 0.675 PLU-7/g flour; Lane 5=DGDG reference material.

Figure 11 shows the construction of expression vector pRX487 from pUC18(L131R) and pLJ48;

Figure 12 shows in graphical form the effect of temperature on stability and activity of a lipolytic enzyme from *Streptomyces sp* L131;

Figure 13 shows in graphical form the substrate specificities of galactolipases from *Streptomyces sp* L131, *Streptomyces avermitilis*, *Corynebacterium efficiens* and *Thermobifida fusca*;

Figure 14 shows the structure of an expression vector pCB5(TF) for expression of *Thermobifida fusca* lipase in *C. glutamicum*;

Figure 15 shows a sequence alignment of L131 and homologues *S. avermitilis* and *T. fusca*;

Figure 16 shows a HPTLC plate of reaction products from enzyme treatment of crude soya oil samples. Lane 1 = control, Lane 2 = 99% crude oil and 1% K371 10% in water, Lane 3 = 98% crude oil and 2% K371 10% in water, Lane 4 = 97% crude oil and 3% K371 10% in water, Lane 5 = 99.7% crude oil and 0.3% Lecitase Ultra™ #3108 1% in water, Lane 6 = 99% crude oil, 0.3% Lecitase Ultra™ #3108 1% in water and 0.7% water. As reference phosphatidylcholine (PC) is analysed; and

Figure 17 shows a HPTLC plate of reaction products from enzyme treatment of crude soya oil samples. Lane 1 = control, Lane 2 = 99% crude oil and 1% K371 10% in water, Lane 3 = 98% crude oil and 2% K371 10% in water, Lane 4 = 97% crude oil

and 3% K371 10% in water, Lane 5 = 99.7% crude oil and 0.3% Lecitase Ultra™ #3108 1% in water, Lane 6 = 99% crude oil, 0.3% Lecitase Ultra™ #3108 1% in water and 0.7% water, together with reference lanes of cholesterol ester, monoglyceride, diglyceride, triglyceride and plant sterol.

5

Example 1: Identification of a galactolipase producing bacterial strain.

Two microbial strains with a similar phenotype coded L130 and L131 were isolated from soil collected in Southern Finland. The 16S RNA genes of these two strains were
10 amplified by standard PCR using oligonucleotide primers 536f (CAGCMGCCGCGGTAATWC) and 1392r-primer (ACGGGCGGTGTGTRC). The resulting PCR fragments were partially sequenced. SEQ ID No.s 1 and 2 are non-enzyme encoding polynucleotides. These sequences are ribosomal 16S RNA genes widely used for taxonomic comparisons. SEQ ID No. 1 and SEQ ID No. 2 were found
15 to have a high similarity. The sequences were then compared to the 16S RNA gene sequences in GenBank. For both isolates the highest homology (97%) was observed with the sequence of a 16S RNA gene from *Streptomyces thermosacchari*. Thus, the strains were named *Streptomyces* sp. L130 and *Streptomyces* sp. L131.

20 **Example 2: Preparation of lipolytic enzyme (galactolipase) samples from strains *Streptomyces* sp. L130 and L131.**

0.5 l of LB medium was inoculated with *Streptomyces* L130 and cultivated on a rotary shaker at 200 rpm and 30°C for 2 days. This culture was used as inoculum for a 10 l
25 fermentor containing the same medium. The cultivation was continued for 3 days at 30°C, 600 rpm stirring rate and 0.5 v/v aeration. The fermentation broth was cleared by centrifugation (15 min at 5000 rpm) and Triton X-100 was added to final concentration of 0.1%. The solution was concentrated using Vivaflow 200 ultrafiltration cell (Vivascience AG, Hannover, Germany) to 300 ml. The concentrate
30 was dialysed against 10 l of 20 mM Tris HCl buffer, pH 7 containing 2 mM CaCl₂ and 2 mM MgCl₂ followed by dialysis against 0.5 l ml of 85% glycerol. The resulting preparations contained 90 U of galactolipase activity assay as defined above (GLU-7).

The strain *Streptomyces* L131 was cultivated under the same conditions and its culture broth was concentrated by the same procedure. The resulting galactolipase preparation contained 70U of activity.

5 Example 3 – Baking experiments

The galactolipases from bacterial isolates L130 and L131 indicated a high activity on polar lipid substrates, galactolipids (DGDG) and phospholipids, (galactolipase and phospholipase activity), equivalent to that of a *Fusarium oxysporum* lipase (Lipopan FTM Novozymes A/S Denmark): however the galactolipase from bacterial isolates
10 L130 and L131 (i.e. the lipolytic enzyme according to the present invention) were found to have no significant activity of triglycerides. This contrasts sharply with the activity *Fusarium oxysporum* lipase – LipopanFTM.

The lipolytic enzymes from bacterial isolates L130 and L131 were prepared as
15 described in Example 2 and were analysed for characterisation of their activity on glycolipids, phospholipids and triglycerides, both in standard assay conditions and within a dough.

Small scale baking experiments and a model dough system. Both enzymes are very
20 active on galactolipids in flour.

Materials and methods.

Three samples of each enzyme were prepared as in Example 3. Each sample was
25 labelled as shown in table 1:

Table 1

| ID | Organism | Label | GLU-7 | PLU-7 |
|-----|--------------------------|---------------------------------|-------|-------|
| 180 | Streptomyces spp L 130 A | Lipolytic enzyme 0,58PLU/mL | 0,95 | 1,31 |
| 181 | Streptomyces spp L 130 B | Lipolytic enzyme 0,44PLU/mL. | 0,91 | 1,31 |
| 182 | Streptomyces spp L 130 C | Lipolytic enzyme 1,8PLU/mL. | 1,21 | 1,53 |
| 183 | Streptomyces spp L 131 A | Lipolytic enzyme 0,54PLU/mL. | 0,63 | 1,29 |
| 184 | Streptomyces spp L 131 B | Lipolytic enzyme 0,64PLU/mL. | 0,84 | 1,16 |
| 185 | Streptomyces spp L 131 C | Lipolytic enzyme 0,85PLU/mL. | 1,35 | 1,17 |

The phospholipase and galactolipase activity of the enzymes were assessed using the phospholipase activity assay (PLU-7) and the galactolipase activity assay (GLU-7) mentioned herein above.

Dough slurry experiment

0.8 gram Wheat flour was scaled in a 12 ml centrifuge tube with lid. 1.5 ml water containing the enzyme was added. The sample was mixed on a Whirley and placed in a heating cabinet at 30 °C for 60 minutes. 6 ml n-Butanol:Ethanol 9:1 was added, and the sample was mixed again until the flour was finely distributed in the solvent. The tubes were the placed in a water bath at 95°C for 10 minutes. Then mixed again and placed on a rotation device 45 rpm, for 45 minutes.

The sample was then centrifuged at 2000 g for 10 minutes. And 2 ml supernatant was transferred to a 10 ml dram glass. The solvent was evaporated at 70 °C under a steam of nitrogen.

The isolated lipids are analysed by GLC.

Gas Chromatography

- 5 Perkin Elmer 8420 Capillary Gas Chromatography equipped with WCOT fused silica column 12.5 m x 0.25 mm ID x 0.1 μ m 5%phenyl-methyl-silicone (CP Sil 8 CB from Crompack).
Carrier: Helium.
- 10 Injection: 1.5 μ L with split.
Detector: FID. 385 °C.
- | | | | | |
|------------------------|----|-----|-----|-----|
| Oven program: | 1 | 2 | 3 | 4 |
| Oven temperature [°C] | 80 | 200 | 240 | 360 |
| Isothermal, time [min] | 2 | 0 | 0 | 10 |
- 15 Temperature rate [°C/min] 20 10 12

Sample preparation: Lipid extracted from 0,2 gram flour was dissolved in 2 mL heptane: pyridine 2:1 containing an internal standard heptadecane, 2 mg/mL. 500 μ L of the sample was transferred to a crimp vial. 100 μ L MSTFA (N-Methyl-N-trimethylsilyl-trifluoroacetamid) was added and the reaction incubated for 15 minutes at 20 90°C.

Calculation: Response factors for monoglycerides, diglycerides, triglycerides, free fatty acid and galactolipids were determined from reference mixtures of these 25 components. Based on these response factors the lipids in the dough were calculated.

Results.

The samples of enzyme from *Streptomyces* were analyzed for phospholipase and galactolipase activity with results shown in table 2. The activity ratio PLU-7/GLU-7 30 was also calculated. The mean ratio for the samples was 1.4 , but with some deviation in some of the samples, which might be explained by analytical deviations.

Table 2

| Sample ID | Organism | GLU-7 | PLU-7 | Ratio PLU-7/GLU-7 |
|-----------|----------|-------|-------|-------------------|
| 180 | L 130 A | 0,95 | 1,31 | 1,4 |
| 181 | L 130 B | 0,91 | 1,31 | 1,4 |
| 182 | L 130 C | 1,21 | 1,53 | 1,3 |
| 183 | L 131 A | 0,63 | 1,29 | 2,0 |
| 184 | L 131 B | 0,84 | 1,16 | 1,4 |
| 185 | L 131 C | 1,35 | 1,17 | 0,9 |

10

Dough experiment.

The activity of the enzyme on wheat lipids was tested in the dough slurry experiment as mentioned under materials and Methods. The isolated lipids from the dough were analysed by GLC as shown in table 3

Table 3. GLC analysis of dough lipids (% based on flour weight). FFA= free fatty acids. MGMG=monogalactosylmonoglyceride. DGMG=digalactosyldiglyceride. MGDG=monogalactosyldiglyceride. DGDG=digalactosyldiglyceride. TRI= triglyceride.

| Sample ID | Enzyme dosage PLU/g flour | FFA | MGMG | DGMG | MGDG | DGDG | TRI |
|-----------|------------------------------|--------|--------|--------|--------|--------|--------|
| 185 | 0,105 | 0,1642 | 0,0042 | 0,0380 | 0,0345 | 0,1520 | 0,5515 |
| 185 | 0,263 | 0,1687 | 0,0130 | 0,0670 | 0,0239 | 0,0941 | 0,5470 |
| 185 | 0,526 | 0,2096 | 0,0121 | 0,0664 | 0,0158 | 0,0617 | 0,5460 |
| 185 | 1,05 | 0,2597 | 0,0036 | 0,0546 | 0,0068 | 0,0303 | 0,5301 |
| 182 | 0,097 | 0,1542 | 0,0051 | 0,0563 | 0,0313 | 0,1148 | 0,5475 |
| 182 | 0,244 | 0,1687 | 0,0159 | 0,0785 | 0,0200 | 0,0566 | 0,5280 |

| | | | | | | | |
|----------------|-------|--------|--------|--------|--------|--------|--------|
| 182 | 0,488 | 0,2095 | 0,0055 | 0,0646 | 0,0098 | 0,0219 | 0,5418 |
| 182 | 0,976 | 0,2581 | 0,0092 | 0,0439 | 0,0043 | 0,0045 | 0,5579 |
| Control | 0 | 0,1529 | 0,0006 | 0,0188 | 0,0440 | 0,1443 | 0,5054 |
| Lipopan FTM | 1,47 | 0,23 | 0,03 | 0,10 | 0,01 | 0,07 | 0,44 |

The results from table 3 and table 4 confirm that the enzymes isolated in the supernatant from fermentation of *Streptomyces* sp L130 and L131 are very active on galactolipids in a dough. The diesters DGDG and MGDG are hydrolyzed to the corresponding monoesters DGMG and MGMG. The results are also illustrated graphically in Figures 6 and 7. These results confirm that both enzymes are very active at low dosage 0-0.2 Units/g flour and corresponding amount of monoester is produced. At higher dosage 0,4-1 Units/gram flour DGDG is further degraded but also some hydrolysis of the monoesters are observed. This may indicate the enzymes are not specific to the position of the fatty acid in the galactolipid molecule.

The activity of the enzymes on triglyceride, as illustrated in Figure 8, is almost not existent. It is therefore concluded that the enzymes tested have no significant effect on triglyceride. This is also in agreement with some experiments conducted on tributyrin as substrate, where no activity was observed.

SUMMARY

A lipolytic enzyme was isolated in the supernatant from fermentation of *Streptomyces* sp.

The lipolytic enzyme was found to have both phospholipase and galactolipase activity, but no significant activity on triglycerides. The ratio of phospholipase: galactolipase activity was approx. 1.4 for the samples tested.

Dough slurry experiments confirms that the enzymes were active on galactolipids in the flour. The enzymes were active in dough at a very low dosage 0-0.2 Units/g flour. Commercial phospholipases like Lipopan FTM (Novozymes A/S, Denmark) need to be dosed in 3-4 times higher dosage in order to obtain the same effect on galactolipids.

- 5 The dough slurry experiments also confirmed that the enzymes from *Streptomyces* sp. had no measurable activity on triglycerides.

Example 4: Cloning of the lipolytic enzyme gene from *Streptomyces* sp. L131.

- 10 The chromosomal DNA was isolated from *Streptomyces* sp. L131 using a modification of a standard method. Bacteria were grown on a rotary shaker in LB medium at 30°C and high aeration (100 ml of medium per 0.5l baffled flask, 200 rpm) to early stationary phase. From 500 ml bacterial culture cells were collected with centrifugation and washed once with lysis buffer (550 mM glucose, 100 mM Tris, 2
15 mM EDTA, pH 8.0).

- Cell pellet was re-suspended in 10 ml of lysis buffer and lysozyme was added to 1 mg/ml. Cells were incubated at 37°C for at least 15 min. The progress of lysozyme digestion was followed by transferring aliquots of bacterial suspension into 1% SDS
20 solution and measuring the absorption of the resulting mixture at 600 nm. The amount of lysozyme and incubation time were adjusted so that at least 70-90% of all cells were lysed as evidenced by the decrease in A₆₀₀. At this point of time, SDS was added to the bacterial suspension to 1% and proteinase K to 0.1 mg/ml. The suspension was incubated at 56°C for 30 min followed by extractions with phenol and chloroform.
25 After chloroform extraction, DNA was precipitated with sodium acetate (0.5M final concentration) and isopropanol (0.6 vol/vol) and the DNA pellet was washed with 70% ethanol, dried in vacuum and dissolved in TE buffer (10 mM Tris, 1 mM EDTA) containing RNase A (0.01 mg/ml).

- 30 The DNA was partially digested with restriction endonuclease *Sau3A* and the hydrolysates fractionated on a 0.8% agarose gel. The 3-10 kb fraction of the *Sau3A*

was isolated from agarose gels by electroelution. This DNA preparation was used to construct a gene library using Stratagene's (LaJolla, USA) ZAP Express/Predigested Vector/Gigapack Cloning Kit (product #239615). Ligation, packaging, amplification of library and its conversion to the phagemid form were carried out according to the protocols provided by Stratagene. Plasmid form of the resulting gene library was screened on indicator plates prepared as follows. 80 ml of sterile LB agar containing 25 mg/l of kanamycin was placed into each 15 cm Petri dish and allowed to solidify. Subsequently, 10 ml top agar layer was added containing 0.5% DGDG and 0.0005% Safranin O. The gene library was plated at a density of approximately 5000 colonies per 15 cm plate. The plates were incubated at 37°C for 24 h followed by a four-day incubation at room temperature. A clone forming red halo on indicator plate was selected from the library and purified by cloning on a new indicator plate.

The plasmid isolated from this clone (named pBK(L131)) was used to re-transform *E. coli* strain XL1-Blue MRF' to kanamycin resistance. All such transformants displayed galactolipase-positive phenotype. pBK(L131) contained an approximately 7.5 kb insert. This insert was sequenced. One sequenced region (SEQ ID No. 3) was found to contain an open reading frame encoding a protein (SEQ ID No. 4) showing homology to a known lipase from *Streptomyces rimosus*. This lipase, a member of so-called GDSX family of lipases/esterases/acyl transferases is only known to be able to hydrolyse neutral lipids and artificial lipase substrates.

A series of deletions and sub-clones of the original insert were constructed and tested for galactolipase activity. It was found that a deletion derivative carrying 3 kb *EcoRI* – *SacI* fragment of the original insert still retains full DGDGse activity. This data correlated well with the results of partial DNA. One area demonstrated homology to known lipases. This area was subsequently sequenced completely. Comparison of this sequence with the GenBank revealed that the closest homologue (58.5%) of the L131 galactolipase that has been biochemically characterised is a lipase from *S. rimosus*, and identified as a lipid:acyl transferase in WO04/064987 and WO04/064537.

Expression of L131 galactolipase in *E. coli*.

The standard pET-system, in which the gene is under control of the T7 phage promoter, was used in to express the L131 galactolipase in *E. coli*.

5

Expression of L131 galactolipase in *Streptomyces lividans*.

The shuttle vector pRX487-5 (Figure 11) (derived from pIJ4987: Kieser T. *et al* Practical *Streptomyces* genetics. The John Innes Foundation, Crowes, Norwich, England (2000)) used for expression of L131 galactolipase in *S. lividans* combines *E. coli* plasmid pUC18 and the *S. lividans* plasmid IJ487. In pRX487-5, the *lac* promoter of pUC18 is placed upstream of promoter-less kanamycin phosphotransferase gene of pIJ487. Indeed, the plasmid transformed *E. coli* not only to ampicillin but also to at least a low level (5 mg/l) of kanamycin resistance. The vector contains unmodified *EcoRI-XbaI* fragment of the chromosomal DNA of *S. thermosacchari* comprising the complete coding sequence of the galactolipase gene, about 160 bp of upstream non-coding sequence and 420 bp of downstream non-coding sequence. All transformants displayed similar levels of galactolipase activity as judged by the halo formation on indicator plates. Similarly, when *S. lividans* carrying pRX487-5 was cultivated at 10 l level and the resulting culture was cloned on indicator plates, all clones appeared to produce equal amounts of galactolipase activity. In small shake-flask cultures inoculated by vegetative cells directly from plates, the transformants typically produced about 10-20 mU/ml of galactolipase activity after 3 days of cultivation. When shake-flask cultures were inoculated with spores of the recombinant *S. lividans*, higher galactolipase activities were measured (about 30 mU/ml), correlating with higher biomass accumulation. In an experiment where *S. lividans* carrying pRX487-5 was grown in fermentor under high aeration conditions and fed-batch mode (see material and methods for details) biomass accumulation reached 170 g/l (wet weight). Accordingly, much higher galactolipase activity – about 1U/ml was detected.

30

Biochemical properties of L131.

Some biochemical properties of L131 were tested. The pH optimum of the enzyme was found to be around 6.5-7.5 (Figure 9). The enzyme exhibited maximum activity towards DGDG at a temperature ~50°C. Above this temperature inactivation occurred, but not sharply, and after 20 min incubation in 90°C, ~10% of residual activity was detected (Figure 12).

Example 5: Expression of *Streptomyces* L131 lipolytic enzyme according to the present invention gene in *E.coli*

The open reading frame of pBK(L131) encoding presumptive lipolytic enzyme according to the present invention was amplified by PCR using primers oL131-5 (GGTGAATTCATGAGATTGACCCGATCCCTGTCGG, sense primer) and oL131-3 (ACTTCTAGAGCGGCCACCGTGACGTACA, anti-sense primer). The amplified DNA fragment was digested with *EcoRI* and *XbaI* and cloned into a *B. subtilis* – *E. coli* shuttle vector pGTK44. This vector has been constructed by substituting the *Sall*-*EcoRI* fragment of plasmid pGTK44 (Povelainen et al., Biochem J. 371, 191-197 (2003)) containing *degQ36* promoter with *EcoRI*-*Sall* fragment of pGT44 (Kerovuo J. et al. Biotechnology Letters 22, 1311-1317 (2000)).

Galactolipase activity was detected in *E. coli* transformed with the resulting plasmid pGTK44(L131) (Figure 5) using indicator plates. Control transformants (containing parent plasmid pGTK44) were galactolipase-negative. Thus, protein sequence represented by SEQ ID No 4 indeed possesses galactolipase activity. The same pair of primers amplified a fragment of the same size (by agarose gel electrophoresis) with chromosomal DNA of *Streptomyces* sp. L130 further confirming earlier observations about close similarity of the two isolated strains and their galactolipase genes.

For expression in *E.coli* under control of the T7 phage promoter, the deduced galactolipase coding region was amplified by PCR using chromosomal DNA of the *Streptomyces* sp. L131 as template and the two oligonucleotide primers (oL131-51

GGTCATGCTAGCATGAGATTGACCCGATCCCTGTCGG and oL131-31
GCATGGATCCGCGGCCACCGTGACGTACA). The PCR product was
digested with *NheI* and *BamHI* and ligated with pET11a (Novagen, USA) vector
digested with the same restriction endonucleases. The ligation mixture was used to
5 transform the *E.coli* strain XL-Blue1 MRF' and 12 different plasmid clones with
restriction patterns corresponding to the structure of pET11(131-51) (Fig. 4) were
isolated. Each plasmid clone was used to separately transform the *E.coli* strain
BL21(DE3) and the resulting transformants were grown on LB-ampicilin containing
galactolipase activity indicator layer (Example 4). Most clones did express active
10 galactolipase. One clone (pET11(131-51)-12) was selected as a source of recombinant
galactolipase for subsequent characterisation.

The enzyme expressed in *E. coli* (labelled #236) was analysed and found to have: 0.33
GLU/ml and 0.36 PLU/ml, when analysed using the GLU-7 assay and PLU-7 assay
15 taught herein.

In liquid culture *E.coli* BL21(DE3) expressed about 2 mU/ml of galactolipase activity
after 40 h cultivation in LB-ampicillin broth (37°C, 200 rpm shaking). Essentially all
of the activity was found in the culture broth. No galactolipase activity was detected
20 in *E.coli* BL21(DE3) transformed with pET11a (Novagen, USA) and cultivated under
the same conditions.

About four litres of galactolipase-containing culture broth culture was concentrated on
a rotary evaporator to about 300 ml and dialysed against 15 l of 20 mM Tris HCl
25 buffer, pH 7 containing 2 mM CaCl₂ and 2 mM MgCl₂. The dialysed material was
again concentrated on a rotary evaporator to about 30 ml and dialysed against 2 l of
50% glycerol. The resulting preparation (18 ml) contained about 100mU/ml of
galactolipase activity.

30 The enzyme expressed in *E. coli* (labelled #236) was also tested in dough. High
activity on galactolipids was observed in dough as can be seen from Figure 10, which
shows a TLC plate.

Example 6: Expression of the lipolytic enzyme according to the present invention gene from *Streptomyces* sp. L131 in different hosts.

5 Construction of the vector pGTK44(L131) has been outlined in the Example 5. Besides *E. coli*, this vector can be used to produce *Streptomyces* L131 lipolytic enzyme according to the present invention in *Bacillus*. Using this vector is only one of many possible ways to express the L131 lipolytic enzyme according to the present invention in *Bacillus*. For example, the *pst* promoter employed in pGTK44(L131)
10 may be replaced by any other strong constitutive or regulated promoter active in *Bacillus*. Many such promoters are known in the art. For example, *degQ36* promoter (Yang M et al. J. Bacteriol. 166, 113-119 (1986)), *cdd* promoter, also known as p43 (Wang PZ, Doi RH. J. Biol. Chem. 259, 8619-8625 (1984), amylase or neutral protease promoters etc. In addition to pGTK44(L131) and other *Bacillus* vectors
15 based on pTZ12 replicon (Aoki T. et al., Mol. Gen. Genet. 208, 348-352 (1987)) any other plasmid vector (e.g pUB110, Gryczan TJ et al. J. Bacteriol. 134, 318-29 (1978) and its derivatives) can be used.

Other preferred hosts for expression of the *Streptomyces* L131 lipolytic enzyme
20 according to the present invention gene are high-GC Gram positive bacteria, in particular, *Streptomyces*, (for example, *S. lividans*, *S. coelicolor*, *S. griseus*, *S. natalensis*, *S. rubiginosus*, *S. olivaceus*, *S. olivochromogenes*, *S. violaceoruber*), In such hosts, the lipolytic enzyme according to the present invention gene can be introduced under its own promoter on a multi-copy vector (e.g. using pIJ110
25 derivatives such as pIJ486, Ward et al. Mol. Gen. Genet. 203, 468-478 (1986)) or placed under control of a strong *Streptomyces* promoter, for example *ermE** (Schmitt-John T, Engels JW. Appl. Microbiol. Biotechnol. 36, 493-498 (1992)) or thiostreptone-inducible *tipA* promoter (Kieser T et al. in Practical Streptomyces Genetics, p. 386, The John Innes Foundation, Norwich UK (2000)).

- In addition to prokaryotic hosts, L131 lipolytic enzyme gene may be expressed in one of the many suitable fungal species. In particular, yeasts such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*, *Hansenula polymorpha* are suitable. In yeast, the lipolytic enzyme gene may be placed under control of any of the
- 5 known strong yeast promoters, such glycolytic promoters (*PGK*, *GDH*, *ENO* etc) phosphate starvation induced promoters such as *PHO5*, the promoters of ethanol/methanol metabolism such as *ADHI* promoter in *S.cerevisiae* or methanol-inducible promoters in *H. polymorpha* or *P. pastoris*.
- 10 When expressing the lipolytic enzyme gene in any host, construction of a synthetic or semi-synthetic gene encoding the sequence of SEQ ID 4 would be advantageous. Likewise, partly or completely synthetic genes may be designed based on sequences available through homology searches *in silico* as explained in Example 4. Such
- 15 sequences, may incorporate a number of useful features that are absent in wild-type lipolytic enzyme genes. For, example, the codon bias can be corrected to better correspond codon preferences of the expression hosts. One special case of codon bias correction useful for all hosts is to convert the GTG initiation codon of SEQ ID No 3 into ATG. Another typical modification obvious for a man skilled in the art is to
- 20 exchange the native *Streptomyces* signal sequence of the L131 lipolytic enzyme with a signal sequence native to or known to be functional in the chosen expression host.
- Previous examples of useful expression systems for L131 lipolytic enzyme focused on using plasmid vectors for the introduction of the lipolytic enzyme gene into the expression host. This is indeed the preferred mode to implement current invention.
- 25 However, an alternative approach of integrating the expression cassette (including promoter, lipolytic enzyme gene coding region and an optional transcription terminator) into a chromosome is also feasible. In particular, multi-copy integration of the expression cassette into the host chromosome would be efficient.
- 30 The recombinant hosts expressing the lipolytic enzyme gene can be, advantageously, mutated to reduce the level of protease activity in the culture broth. The cultivation of any of such recombinant hosts can be carried out in the presence of compounds

stabilising the enzyme. Such compounds may be various proteins (e.g. casein, peptone of serum albumin) or different lipids, lysolipids or detergents (e.g. galactolipids, mono- and diacylglycerols or Triton X-100).

5 **Example 7** Acyl-transferase activity of *Streptomyces* L131 lipolytic enzyme and its derivatives

Some lipases may also possess acyl-transferase activity. In particular, some members of the GDSX family, for example, *Aeromonas hydrophila* acyltransferase (P10480)
10 (taught in copending International Application No. PCT/IB2004/000655) have high acyl-transferase activity. Thus, *Streptomyces* L131 lipolytic enzyme may be predicted to have also the acyl-transferase activity as well. This activity can be further enhanced through random mutagenesis /directed evolution. Moreover, since *A. hydrophila* acyl-transferase and *Streptomyces* L131 lipolytic enzyme share the same overall protein
15 fold, combining the substrate specificity of *Streptomyces* L131 lipolytic enzyme with high transferase efficiency of the *Aeromonas* enzyme is possible. This combination may be achieved through the known techniques of targeted mutagenesis/protein design or by gene shuffling.

20 **Example 8** Identification of alternative lipolytic enzymes from other *Streptomyces* species.

The GDSX family of esterase's (Upton C, Buckley JT. Trends Biochem. Sic. 20, 178-179 (1995), pfam00657.11) is a group of esterases / lipases /acyl transferases sharing a
25 specific sequence motif around the active site serine (GDSX where X is a hydrophobic amino acid residue). This group of enzymes is also known as lipase family II (Arpigny JL, Jaeger K-E. Biochem. J. 343, 177-183 (1999)). Although this family includes many different types of esterases, lipases and acyl-transferases, the lipolytic enzyme according to the present invention is a GDSX enzyme.

Thus, the sequences taught in the present invention of the *Streptomyces* sp. L131 lipolytic enzyme (galactolipase) can be used *in silico* to identify other galactolipases from other species of *Streptomyces*.

- 5 To determine if a protein has the GDSX motif according to the present invention, the sequence is preferably compared with the hidden markov model profiles (HMM profiles) of the pfam database.

Pfam is a database of protein domain families. Pfam contains curated multiple
10 sequence alignments for each family as well as profile hidden Markov models (profile HMMs) for identifying these domains in new sequences. An introduction to Pfam can be found in Bateman A *et al.* (2002) *Nucleic Acids Res.* 30; 276-280. Hidden Markov models are used in a number of databases that aim at classifying proteins, for review see Bateman A and Haft DH (2002) *Brief Bioinform* 3; 236-245.

15

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=12230032&dopt=Abstract

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=11752314&dopt=Abstract

20

For a detailed explanation of hidden Markov models and how they are applied in the Pfam database see Durbin R, Eddy S, and Krogh A (1998) *Biological sequence analysis; probabilistic models of proteins and nucleic acids*. Cambridge University Press, ISBN 0-521-62041-4. The Hammer software package can be obtained from
25 Washington University, St Louis, USA.

Alternatively, the GDSX motif can be identified using the Hammer software package, the instructions are provided in Durbin R, Eddy S, and Krogh A (1998) *Biological sequence analysis; probabilistic models of proteins and nucleic acids*. Cambridge
30 University Press, ISBN 0-521-62041-4 and the references therein, and the HMMER2 profile provided within this specification.

The PFAM database can be accessed, for example, through several servers which are currently located at the following websites.

<http://www.sanger.ac.uk/Software/Pfam/index.shtml>

5 <http://pfam.wustl.edu/>

<http://pfam.jouy.inra.fr/>

<http://pfam.cgb.ki.se/>

10 The database offers a search facility where one can enter a protein sequence. Using the default parameters of the database the protein sequence will then be analysed for the presence of Pfam domains. The GDSX domain is an established domain in the database and as such its presence in any query sequence will be recognised. The database will return the alignment of the Pfam00657 consensus sequence to the query sequence.

15

Preferably when aligned with the Pfam00657 consensus sequence the lipolytic enzyme for use in the compositions/methods of the invention have at least one, preferably more than one, preferably more than two, of the following, a GDSx block, a GANDY block, a HPT block. Suitably, the lipolytic enzyme may have a GDSx block and a GANDY block. Alternatively, the enzyme may have a GDSx block and a HPT block. Preferably the enzyme comprises at least a GDSx block.

20

The pfam00657 GDSX domain is a unique identifier which distinguishes proteins possessing this domain from other enzymes.

25

In addition or as an alternative thereto, alternative lipolytic enzymes from other *Streptomyces* species can be identified by conducting a sequence identity comparison and/or hybridisation with one or more of the PCR sequence fragments shown as SEQ ID No. 1 or SEQ ID No. 2. Suitably, the comparisons may be carried out with fragments comprising over 15 nucleotides of SEQ ID No. 1 or SEQ ID No. 2, preferably with fragments comprising over 20 nucleotides of SEQ ID No. 1 or SEQ ID No. 2. Suitably,

30

the complete sequences shown as SEQ ID No. 1 or SEQ ID No. 2 could be used. Preferably, the hybridisation is carried out at high or very high stringency conditions. Nucleotide sequences having at least 80%, preferably at least 85%, at least 90%, at least 95%, at least 97%, at least 98% or at least 99% identity to SEQ ID No. 1 or SEQ ID No. 2
5 indicate strains of *Streptomyces* which may be sources of the lipolytic enzyme, i.e. the galactolipase, according to the present invention.

Example 9: Identification of galactolipases for use in the methods and uses of the present application

10

As mentioned above, the sequence of the novel *Streptomyces thermosacchari* L131 offers for the possibility for *in silico* identification of new family II galactolipases. In this regard, one particular region which may be of particular interest is the GDSX motif.

15

The GDSX motif is comprised of four conserved amino acids. Preferably, the serine within the motif is a catalytic serine of the lipid acyltransferase enzyme. Suitably, the serine of the GDSX motif may be in a position corresponding to Ser-16 in *Aeromonas hydrophila* lipolytic enzyme taught in Brumlik & Buckley (Journal of Bacteriology
20 Apr. 1996, Vol. 178, No. 7, p 2060-2064).

To determine if a protein has the GDSX motif, the sequence is preferably compared with the hidden markov model profiles (HMM profiles) of the pfam database. As mentioned in Example 8, pfam is a database of protein domain families. Thus, the
25 pfam database may also be used to identify suitable enzymes from genera other than *Streptomyces*.

Alternatively, the GDSX motif can be identified using the Hammer software package, the instructions are provided in Durbin R, Eddy S, and Krogh A (1998) Biological
30 sequence analysis; probabilistic models of proteins and nucleic acids. Cambridge University Press, ISBN 0-521-62041-4 and the references therein, and the HMMER2 profile provided within this specification.

Preferably, the lipolytic enzyme in accordance with the present invention comprises the GDSX motif.

- 5 When aligned to either the pfam Pfam00657 consensus sequence (as described in WO04/064987) and/ or the L131 sequence herein disclosed (SEQ ID No 4)
- i) The galactolipase/lipid acyl-transferase enzyme of the invention, or for use in methods of the invention, has preferably a GDSx motif, more
10 preferably a GDSY motif
- and/or
- ii) The galactolipase/lipid acyl-transferase enzyme of the invention, or for use in methods of the invention, has preferably a GANDY block, more
15 preferably a GANDY block comprising amino GGNDx, more preferably GGNDA or GGNDL
- and/or
- 20 iii) The enzyme of the invention, or for use in methods of the invention, has preferable an HTP block.
- and preferably
- 25 iv) The galactolipase/lipid acyl-transferase enzyme of the invention, or for use in methods of the invention, has preferably a GDSY motif and a GANDY block comprising amino GGNDx, preferably GGNDA or GGNDL, and a HTP block (conserved histadine).
- 30

In this regard, the inventors identified a homologous sequence to *Streptomyces* L131 which did not comprise a GDSX motif: namely *Novosphingobium aromaticivorans* (NAL)

Novosphingobium\aromaticivorans\ GDSx 284 aa

SEQ ID No. 10

5 ZP_00094165

1 mgqvklfarr capvllalag lapaatvare aplaegaryv algssfaagp gvgnpapgsp
61 ercgrgtlly phllaealki dlvdatscga tthhvlgpwn evppqidsvn gdirivltti
121 ggndvsvfvg ifaaacekma spdprcgkwr eiteewqad eermrsivrq iharaplarv
10 181 wvdyitvip psgtcaamai spdrlaqsrs aakrlarita rvareegasl lkfshisrrh
241 hpcsakpwsn glsapaddgi pvhpnrigha eaaaaivklv klmk
/

15 SEQ ID No. 11

15 1 tgccggaact caagcggcgt clagccgaac tcatgcccca aagcgcgtgg caclatcccg
61 aagaccaggt ctcggacgcc agcagagcgc tgalggccgc cgaaatcacg cgcgaacagc
121 tciaccgcca gctccacgac gagctgccct atgacagtac cgtacgtccc gagaagtacc
181 tccatcgcaa ggacggctcg atcgagatcc accagcagat cgtgaltgcc cgcgagacac
20 241 agcgtccgat cgtgctgggc aaggggtggcg cgaagatcaa ggcgatcggg gaggccgcac
301 gcaaggaact ttcgcaattg ctcgacacca aggtgcacct gttcctgcat gtgaaggtcg
361 acgagcgtcg ggccgacgcc aaggaaatct acgaggaaat cggccctcga tgggtcaagt
421 gaagctcttc gcgcgccgct gcgccccagt actctcgcct ctgcccgggc tggctccggc
481 ggctacggic gcgcgggaag caccgctggc cgaagggcgc cgttacgttg cgtgggaag
25 541 ctccctgcc gcaggtccgg gcgtggggcc caacgcgcc ggatcgcggc aacgctcgg
601 ccggggcacg ctcaactacc cgcacctgct cgcgaggcgc ctcaagctcg aictcgtcga
661 tgcgacctc agcgcgcgca cgaccacca cgtgctgggc cctggaacg aggtccccc
721 tcagatcgac agcgtgaatg gcgacaccg cctcgtcacc ctgaccatcg gcggaaacga
781 tgtgtcgttc gtcggcaaca tctcgcgc cgtctgcgag aagatggcgt cgcgccatcc
30 841 gcgctcggc aagtggcgg agatcaccga ggaagagtgg caggccgacg aggagcggat
901 gcgctccalc gtacgccaga tccacgccg cgcgcctc ccccgggtgg tgggtgctga
961 ifacatcacg gtctcggc calcaggcac ttgcgtgcc atggcgattt cgcgggaccg
1021 gctggcccag agccgcagcg ccgcgaaacg gctgcccgg ataccgcac gggctcgcg
1081 agaagagggg gcatgctgc tcaagttc gcafatctcg cgcggcacc atccatgctc
35 1141 tgccaagccc tggagcaacg gccttccg cccggccgac gacggcatcc cggttccatcc
1201 gaaccggctc ggacatgctg aagcggcagc ggcgctggc aagctgtga aattgalgaa
1261 gtgactacty cactgattc aaatagtatt gcctgtcagc ttccagccc ggalgtgtc
1321 agcgcaacag aaactgtcc gtaatgatt gatggttat gtcgctcga aattgcccgc
1381 gaagggaaac ggcgcgtcgc tcgttaactg cctgggtgca gcagtgacgg agcgcgtgga
40 1441 tgagtatc tggcgggtgc atcgggtlac gcgccccat tccatgcct gtacgcgccg
//

This enzyme comprises the sequence "GSSF" as opposed to GDSX.

45 When tested it was found that this enzyme does not comprise glycolipase activity in accordance with the present invention.

Therefore, the GDSx motif may be important when attempting to identify other suitable galactolipases.

Notably, the enzyme from *S. rimosus* that has been purified and characterised biochemically and shows about 56% sequence homology to *Streptomyces* L131 (Abramić M, et al. (1999); Vujaklija D. et al. (2002)) is known to hydrolyse neutral lipids such as triolein or nitrophenyl esters of fatty. The enzyme from *S. rimosus* may also hydrolyse galactolipase in accordance with the present invention. Similarly, two other *Streptomyces* species for which genome sequence data is available – *S. coelicolor* A2(3) and *S. avermitilis* may contain enzymes having galactolipase activity, for example (NP_625998 and NP_827753) are currently annotated in GenBank as “putative secreted hydrolases” .

Many other useful homologues of *Streptomyces* L131 galactolipase can be identified by a similar approach. Suitable galactolipase/lipid acyl-transferase enzyme enzymes for use in the methods of the invention may be identified by alignment to the L131 sequence using Align X, the Clustal W pairwise alignment algorithm of VectorNTI using default settings.

Alternatively, suitable galactolipase for use in the methods of the invention may be identified by alignment to the pfam Pfam00657 consensus sequence (as described in WO04/064987).

Figure 15 shows an sequence alignment of the L131 and homologues from *S. avermitilis* and *T. fusca*. Figure 15 illustrates the conservation of the GDSx motif (GDSY in L131 and *S. avermitilis* and *T. fusca*), the GANDY box, which is either GGND A or GGND L, and the HPT block (considered to be the conserved catalytic histadine). These three conserved blocks are highlighted in Figure 15.

When aligned to either the pfam Pfam00657 consensus sequence (as described in WO04/064987) and/ or the L131 sequence herein disclosed (SEQ ID No 4) it is possible to identify three conserved regions, the GDSx block, the GANDY block and the HTP block (see WO04/064987 for further details).

Example 10: Gene cloning and construction of expression vectors

Corynebacterium efficiens DSM 44549, *Thermobifida fusca* DSM 43792 and
5 *Streptomyces avermitilis* DSM46492 were used for isolating the genes homologous to
the galactolipase gene of *S. thermosacchari* L131.

The strains accorded with a DSM number are deposited and publically available with
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM).

10

Escherichia coli strains XL-Blue MRF', BL21(DE3) (Novagen) and S17-1 (Simon R.
et al., 1983), *Bacillus subtilis* BD170, *Streptomyces lividans* strain 1326 (John Innes
Centre), *Corynebacterium glutamicum* DSM20300 were used as the hosts for
heterologous expression. The strain of *Aeromonas salmonicida* (DSM 12609) was also
15 used as an expression host.

S. thermosacchari L131, *Citrobacter freundii* P3-42 and *Enterobacter nimipressuralis*
P1-60 were isolated in our laboratory from natural environment and taxonomically
identified by 16S rRNA gene sequencing.

20

The following culture media were used in this study. LB (5 g/l yeast extract, 10 g/l
tryptone, 10 g/l NaCl, pH 7.0), 2xYT (10g/l NaCl, 10g/l yeast extract, 16 g/l tryptone)
were used for cultivation of *E.coli* and other Gram-negative bacteria. Nutrient broth (3
g/l beef extract, 5 g/l peptone, pH 7.0) was used for growing *C. efficiens* and *N.*
25 *aromaticivorans*, YM-broth (3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 10 g/l
dextrose, pH 7.0) was used for cultivation of *S. avermitilis*, Medium 65 (4 g/l glucose,
4 g/l tryptone, 10 g/l malt extract, 2 g/l CaCO₃, pH 7.2) was used for *T. fusca*.

DNA isolation.

30

Standard alkaline lysis procedure combined with Qiagen column purification method
was used for plasmid isolation. One exception was the preparative isolation of

plasmid DNA from *Streptomyces*. In this case, equilibrium centrifugation in CsCl gradient was used as the final purification step.

Methods for introduction of DNA into microbial strains.

5

Both *E. coli* and *C. glutamicum* strains were transformed by electroporation using 1 mm cuvettes and the following electroporation parameter settings: 1800V, 25°F, 200 µl *B. subtilis* BD170 was transformed by "Paris" method based on natural competence (Harwood C.R. and Cutting S.M., 1990). *Streptomyces lividans* was transformed by protoplast method (Kieser T. et al. , 2000). DNA was introduced into *A. salmonicida* by conjugation with *E. coli* using filter mating method of Harayama et al. (1980).

10

Construction of rifampicin-resistant mutant of *A. salmonicida*.

15 About 10^8 cells from overnight culture of *A. salmonicida* DSM12609 were plated on a series of LB agar plates containing 5-30 mg/l rifampicin. The plates were irradiated by short wave UV light using SpectroLinker XL-1500 device (Spectronics Corp. USA). The radiation dose was 4-6 J/M². The plates were incubated at 30°C for 2 days. Several colonies growing on 30 mg/l rifampicin were selected and additionally tested
20 on 50 mg/l rifampicin. One clone resistant to 50 mg/l rifampicin (named R1) was chosen for subsequent work.

Construction of *E. coli* expression vectors for L131 galactolipase homologues.

25 The lipase gene of *Streptomyces avermitilis* was amplified by PCR using chromosomal DNA as template and the two oligonucleotide primers oSAL-5 (GGG AATCCATATGAGACGTTCCCGAATTACG) and oSAL-3 (GCATGGATCCGG TGACCTGTGCGACGG). For amplification of lipase genes of *Thermobifida fusca* and *Corynebacterium efficiens* the oligonucleotide primers used were oTFL-5
30 (GGGAATTCCATATGGGCAGCGGACCACGTG) and oTFL-3 (GCATGGATCCGACACGCACGGCTCAACG), oCEL-5 (GGGAATTCC ATATGAGGACAACGGTCATCG) and oCEL-3 (GCATGGATCCGGC

ATCGGGCTCATCC), respectively. The PCR products were digested with *Nde*I and *Bam*HI and ligated with pET11a (Novagen, USA) vector digested with the same restriction endonucleases.

5 L131 galactolipase expression vector for *S. lividans* was constructed as follows. Plasmid pUC18(L131RX) that contains the 1.37 kb *Eco*RI-*Xba*I fragment of the original cloned DNA fragment carrying L131 lipase gene (pBK(L131)) was digested with *Eco*RI and ligated with *Eco*RI digested pIJ487 (Kieser *et al.*, 2000). This ligation leads to the formation of the two recombinant plasmids differing in relative
10 orientation of pIJ487 and pUC18(L131RX). For subsequent work a variant where *lac* promoter of the pUC18 is flanking the promoter-less *neo*^R gene of pIJ487 has been selected based on restriction analysis. This construction was named pRX487-5 (Figure 11). Besides ampicillin resistance, this plasmid also confers *E. coli* the resistance to at least 3 mg/l kanamycin. The protoplasts of *S. lividans* 1326 were transformed with
15 0.1-10 µg of pRX487-5 to thiostreptone (1.2 mg/l) and kanamycin (5 mg/l) resistance. These transformants produced active galactolipase as judged by the DGDG-safranin indicator plate assay. The transformants were plated on SM plates (Kieser *et al.*, 2000) supplemented with 5 mg/ml of kanamycin and allowed to sporulate. The resulting spores were used for inoculating shake flask and fermentor cultures.

20

Construction of expression vectors for *Corynebacterium glutamicum*.

All expression vectors used in this work are based on the plasmid pCB5 which is a shuttle vector carrying *C. glutamicum* replicon from plasmid pSR1 (Yoshihama *et al.*,
25 1985) and ColE1 replicon from *E. coli*. The promoter that is used in this vector is derived from the *cop1* gene encoding the major secreted protein of *C. glutamicum* – PS1. Enzymes were expressed from their native genes including unmodified signal peptides, e.g. *T. fusca* (Figure 14).

FERMENTATION CONDITIONS

Fermentation of lipase-producing *Streptomyces* strains.

5

In shake flasks, lipase-producing recombinant *S. lividans* strains were grown in a medium containing (per litre) 10 g peptone, 5 g yeast extract, 2 g K_2HPO_4 and 10 g glucose (pH 7.0) supplemented with appropriate antibiotics: thiostreptone was used at 1,2 mg/l, kanamycin at 20 mg/l, chloramphenicol at 1,5 mg/l and erythromycin at 1,5 mg/l. Spore suspensions produced by growing the transformants on SM plates were used to start the cultivations.

For fed-batch fermentations, Braun Biostat E fermentor (10 l) was used. The initial medium (7 l), contained (per litre): peptone 20 g, yeast extract, 10 g, glucose 20 g and appropriate antibiotics as described above (except for thiostreptone, which was not used in 10 l cultures). The cultivation was conducted at 30°C, constant 10 l/min aeration and 600 rpm stirring rate. Inocula (2 x 250 ml per fermentation) were grown in 2 l Erlenmeyer flasks as described in the previous paragraph. The fermentation was carried out in batch mode for 18-20 h after which time, a solution containing 30 % glucose and 12,5 % peptone was fed to the fermentor culture at a rate of 0,5 ml/min. Samples (30 ml) of the culture were withdrawn aseptically twice a day.

Fermentation of recombinant *C. glutamicum* strains.

25 Shake-flask cultures of *C. glutamicum* were grown in LB containing 50 mg/l kanamycin at 30°C and 200 rpm agitation rate.

30

Fermentation of recombinant *A. salmonicida* strains.

In shake flasks, the recombinant *A. salmonicida* strains were cultivated in 2xYT medium supplemented with streptomycin and kanamycin (at 25mg/l). To induce *tac* promoter, IPTG (1-5 mM) or lactose (1-10%) were added to the growth medium.

Two sets of conditions for production of recombinant acyl-transferase in *A. salmonicida* were tested at fermentor scale. In the first experiment, the initial medium (7 l) was 2xYT supplemented with 2% glucose, 50 mg/l of kanamycin and 50 mg/l of streptomycin and the feeding solution (3 l) contained 25% glucose, 10% tryptone and 5 %yeast extract, 100 mg/l of both kanamycin and streptomycin. Cultivation was carried out at 10 l/min aeration, 600 rpm stirring rate and 28°C. The pH was adjusted to 7.5 by 25% NH₃ and 10% phosphoric acid. The fermentor was inoculated with 0.5 l of overnight culture of *A. salmonicida* and grown in batch mode for 24 h. At this point IPTG was added to 5 mM and the feeding was started at a rate of 50 ml/h.

In the second experiment, the initial medium was modified by substituting glucose with lactose. Feeding solution was 2 l of 20% lactose. The fermentation temperature was increased to 30°C and the pH of the culture medium decreased to 7.0. Inoculation was done as in the first experiment and the feeding (100 ml/h) was started after 20 h of cultivation in the initial medium

ENZYME ASSAYS**25 Safranine plate screening method.**

In safranine plate screening the bottom layer contained culture medium + additive, 1.5% agarose and 0.002% safranine (0.2% stock solution in water, sterile filtered) and the top layer 0.7% agarose, 1% DGDG and 0.002% safranine.

Determination of galactolipase activity (glycolipase activity assay (GLU-7)):**Substrate:**

- 0.6% digalactosyldiglyceride (Sigma D 4651), 0.4% Triton-X 100 (Sigma X-100)
5 and 5 mM CaCl₂ was dissolved in 0.05M HEPES buffer pH 7.

Assay procedure:

- 400 µL substrate was added to an 1.5 mL Eppendorf tube and placed in an Eppendorf
Thermomixer at 37°C for 5 minutes. At time t= 0 min, 50 µL enzyme solution was
added. Also a blank with water instead of enzyme was analyzed. The sample was
10 mixed at 10x100 rpm in an Eppendorf Thermomixer at 37°C for 10 minutes. At time
t=10 min the Eppendorf tube was placed in another thermomixer at 99°C for 10
minutes to stop the reaction.

Free fatty acid in the samples was analyzed by using the NEFA C kit from WAKO
GmbH.

- 15 Enzyme activity GLU at pH 7 was calculated as micromole fatty acid produced per
minute under assay conditions

Determination of phospholipase activity (phospholipase activity assay (PLU-7)):**20 Substrate**

0.6% L-α Phosphatidylcholine 95% Plant (Avanti #441601), 0.4% Triton-X 100
(Sigma X-100) and 5 mM CaCl₂ was dispersed in 0.05M HEPES buffer pH 7.

Assay procedure:

- 400 µL substrate was added to an 1.5 mL Eppendorf tube and placed in an Eppendorf
25 Thermomixer at 37°C for 5 minutes. At time t= 0 min, 50 µL enzyme solution was
added. Also a blank with water instead of enzyme was analyzed. The sample was
mixed at 10x100 rpm in an Eppendorf Thermomixer at 37°C for 10 minutes. At time
t=10 min the Eppendorf tube was placed in another thermomixer at 99°C for 10
minutes to stop the reaction.

- 30 Free fatty acid in the samples was analyzed by using the NEFA C kit from WAKO
GmbH.

Enzyme activity PLU-7 at pH 7 was calculated as micromole fatty acid produced per minute under assay conditions.

Spectrophotometric assay with *p*-nitrophenyl palmitate (*p*NPP).

5

Lipase activity was measured with a spectrophotometric assay at 30°C with *p*NPP as substrate, by using 50 mM Tris-Maleate buffer (pH 6.5) with 0,4 % Triton X-100 and 0,1 % gum Arabic. The substrate stock solution (100 mM) was prepared in dioxane. The kinetic measurement was started by addition of enzyme to the reaction mixture.

10 To evaluate the initial hydrolytic activity, the increase in absorption at 410 nm was followed with Spectramax plate reader every 20 s for 20 min. One unit of lipase activity was defined as the amount of enzyme that liberated 1 μ mol of *p*-nitrophenol per min. The activity toward other *p*-NP esters was measured in the same manner, by using 1 mM each substrate. (Abramic M. et al. (1999))

15

Determination of effects of pH and temperature on lipase activity.

For the determination of the effect of pH on enzymatic activity, it was measured over a range of pH 2-10 by using the galactolipase activity assay except that the buffers used

20 in the experiment were as follows: pH 2-3.5 Glycine-HCl; pH 4-5 NaOAc; pH 5.5-7.5 Tris-Maleate; pH 7.5-9 Tris-HCl; pH 10 CAPS.

The effect of temperature on galactolipase stability was determined by incubating aliquots of enzyme for 20 min at various temperatures (22°C-90°C) following

25 incubation on ice for 60 min. Residual activity was analysed by galactolipase activity assay.

For detection of optimal temperature for galactolipase activity, the usual assay mixture was equilibrated at the required temperature (the range 20°C-70°C) and 2 or 4 μ l of

30 enzyme was added to start the reaction. The activity was analysed by galactolipase activity assay, but using a shorter period of time (20 min).

Example 11: Characterisation of galactolipase candidates from biodiversity study.

The sequence of *Streptomyces thermosacchari* L131 galactolipase offers for the
5 possibility for *in silico* identification of new family II galactolipases.

Many other useful homologues of *Streptomyces* L131 galactolipase can be identified,
for example, "hypothetical protein" from *Thermobifida fusca* (ZP_00058717) and
"hypothetical protein" from *Corynebacterium efficiens* (NP_738716).

10

We cloned and expressed 3 homologues of *Streptomyces* L131 galactolipase: the genes
of *Streptomyces avermitilis* (SAL), *Thermobifida fusca* (TFL), and *Corynebacterium*
efficiens (CEL). All genes were expressed in *E. coli* by using pET expression system.
The recombinant *E. coli* strains were first analysed using DGDG -indicator plates with
15 safranine and the enzymes of *S. avermitilis*, *T. fusca* and *C. efficiens* were found to
have galactolipase activity.

The enzymes showing galactolipase activity were further examined. Substrate
specificities of those galactolipase candidates were studied (Figure 13). The activity of
20 candidate enzymes towards DGDG, lecithin, olive oil, nitrophenyl butyrate,
nitrophenyl decanoate (NP-D) and nitrophenyl palmitate was tested. The enzymes
were found to have very different substrate specificity profiles. Acyl-transferase
activity was tested in an assay using NP-D as substrate and quantifying both the
release of nitrophenol and free fatty acids by NEFA kit. Preliminary data suggests
25 that at least the enzyme from *Thermobifida fusca* has transferase activity towards
glycerol and glucose.

Thermo-stability of galactolipase candidates was tested. It was found that the
Corynebacterium efficiens enzyme was the most thermostable while the enzyme of
30 *Streptomyces avermitilis* was the most thermo-sensitive.

Example 12: *Streptomyces thermosacchari* L131 degumming trial

5

A phospholipase from *Streptomyces thermosacchari* L131 was tested in crude soya oil.

Materials and methods

10

K371: *Streptomyces thermosacchari* L131 enzyme expressed in *S. lividans* freeze dried on starch.

(Activity: 108 PLU-7/g).

Lecitase Ultra (#3108) from Novozymes, Denmark

15

Cholesterol ester, Fluka 26950

Plant Sterol: Generol 122 N from Henkel, Germany

Crude soya oil from The Solae Company, Aarhus Denmark

Lecithin: L- α Phosphatidylcholine 95% Plant (Avanti #441601)

20

Phospholipase activity

The phospholipase assay was the same as that used in Example 10.

25 **HPTLC**

Applicator: Automatic TLC Sampler 4, CAMAG

HPTLC plate: 20 x 10 cm, Merck no. 1.05641. Activated 30 minutes at 160°C before use.

30 Application: 1 μ l of a 8% solution of oil in buffer was applied to the HPTLC plate using Automatic TLC applicator.

Running buffer 4: Chloroform:Methanol:Water 75:25:4

Running buffer 5: P-ether : Methyl-tert-butyl ketone : Acetic acid 70:30:1

Application/Elution time:

5 Running buffer 4: 20 min

Running buffer 5: 10 min

TLC Development

- 10 The plate was dried in an oven for 10 minutes at 160°C, cooled, and dipped into 6% cupri acetate in 16% H₃PO₄. Dried additionally 10 minutes at 160°C and evaluated directly.

Degumming experiment

15

Streptomyces thermosacchari L131 (K371) was used for degumming studies in the formulations shown in table 4.

- 20 The samples were placed at 40°C for 18 hours with agitation, after which time a sample was collected for HPTLC analysis by dissolving the sample in Chloroform :Methanol 2:1

Table 4. Degumming of crude soya oil with *Streptomyces thermosacchari* L131
And Lecitase Ultra™

| Lane | | 1 | 2 | 3 | 4 | 5 | 6 |
|------------------------------------|---|----|----|----|----|------|-----|
| Crude soya oil | % | 99 | 99 | 98 | 97 | 99,7 | 99 |
| K371, 10% in water | % | | 1 | 2 | 3 | | |
| Lecitase Ultra™ #3108, 1% in water | % | | | | | 0,3 | 0,3 |
| Water | % | 1 | 0 | 0 | 0 | | 0,7 |

25

The results from the HPTLC analysis are shown in Figure 16 and Figure 17.

Figure 16 shows TLC plate (Buffer 4) of reaction products from enzyme treatment of crude soya oil samples according to table 4. As referenced, phosphatidylcholine (PC) was also analysed. Phosphatidylethanolamine (PE) and lysophosphatidylcholine (LPC) are also indicated.

5

The TLC results in Figure 16 clearly show that phosphatidylcholine was completely removed by adding *Streptomyces thermosacchari* L131 to the oil. Only the lowest dosage (lane 2) did not completely hydrolyse the phospholipids. Lecitase Ultra™ also hydrolysed the phospholipids in the oil when 5% water was available (Lane 6) but
10 without adding extra water (Lane 5) only part of the phospholipids were hydrolysed.

Figure 17 shows TLC (Buffer 5) of reaction products from enzyme treatment of crude soya oil samples according to table 4. As referenced, cholesterol ester, monoglyceride, diglyceride, triglyceride and plant sterol. Free fatty acid (FFA) is also indicated

15

The results shown in figure 17 indicate that the hydrolysis of phospholipids is coincident with the formation of free fatty acid.

20 **Conclusion.**

The results confirm that *Streptomyces thermosacchari* L131 effectively hydrolyses phospholipids in crude soya oil and is a suitable alternative enzyme for degumming of
25 plant oils.

25

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and
30 spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed,

various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

References

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BUDAPEST TREATY ON THE INTERNATIONAL
 RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
 FOR THE PURPOSES OF PATENT PROCEDURE

Danisco Intellectual Assets
 Danisco A/S
 Langebrogade 1
 DK-1001 Copenhagen
 Denmark

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
 issued pursuant to Rule 7.1 by the
 INTERNATIONAL DEPOSITARY AUTHORITY
 identified at the bottom of this page

NAME AND ADDRESS OF DEPOSITOR

| | |
|---|--|
| I IDENTIFICATION OF THE MICROORGANISM | |
| Identification reference given by the DEPOSITOR: <i>Streptomyces</i> sp. L130 | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 41226 |
| II SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION | |
| The microorganism identified under I above was accompanied by: | |
| <input type="checkbox"/> a scientific description <input checked="" type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable) | |
| III RECEIPT AND ACCEPTANCE | |
| This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 23 June 2004 (date of the original deposit) ¹ | |
| IV RECEIPT OF REQUEST FOR CONVERSION | |
| The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion) | |
| V INTERNATIONAL DEPOSITARY AUTHORITY | |
| Name: NCIMB Ltd. Address: 23 St Machar Drive Aberdeen AB24 3RY Scotland, UK. | Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s):  Date: 28 June 2004 |

¹ Where Rule 6/4(d) applies, such date is the date on which the status of International Depositary Authority was acquired.

**BUDAPEST TREATY ON THE INTERNATIONAL
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| Danisco Intellectual Assets Danisco A/S Langebrogade 1 DK-1001 Copenhagen Denmark |
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VIABILITY STATEMENT
 issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
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**NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED**

| I. DEPOSITOR | II. IDENTIFICATION OF THE MICROORGANISM |
|--|--|
| Name: AS ABOVE Address: | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 41226 Date of the deposit or of the transfer ¹ : 23 June 2004 |
| III. VIABILITY STATEMENT | |
| The viability of the microorganism identified under II above was tested on 25 June 2004 ² . On that date, the said microorganism was: | |
| <input checked="checked" type="checkbox"/> ³ viable | |
| <input type="checkbox"/> no longer viable | |

¹ Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED⁴

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd.,
Address: 23 St Machar Drive
Aberdeen
AB24 3RY
Scotland

Signature(s) of person(s) having the power
to represent the International Depositary
Authority or of authorised official(s):



Date: 28 June 2004

⁴ Fill in if the information has been requested and if the results of the test were negative.


**BUDAPEST TREATY ON THE INTERNATIONAL
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FOR THE PURPOSES OF PATENT PROCEDURE**

Danisco Intellectual Assets
Danisco A/S
Langebrogade 1
DK-1001 Copenhagen
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NAME AND ADDRESS OF DEPOSITOR

| | |
|---|--|
| I. IDENTIFICATION OF THE MICROORGANISM | |
| Identification reference given by the DEPOSITOR: <i>Streptomyces</i> sp. L131 | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 41227 |
| II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION | |
| The microorganism identified under I above was accompanied by: | |
| <input type="checkbox"/> a scientific description | |
| <input checked="" type="checkbox"/> a proposed taxonomic designation | |
| (Mark with a cross where applicable) | |
| III. RECEIPT AND ACCEPTANCE | |
| This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 23 June 2004 (date of the original deposit) ¹ | |
| IV. RECEIPT OF REQUEST FOR CONVERSION | |
| The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion) | |
| V. INTERNATIONAL DEPOSITARY AUTHORITY | |
| Name: NCIMB Ltd. Address: 23 St Machar Drive Aberdeen AB24 3RY Scotland, UK. | Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s):  Date: 28 June 2004 |

¹ Where Rule 6/4(d) applies, such date is the date on which the status of International Depositary Authority was acquired.
Form BP/4 (sole page)

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 TO WHOM THE VIABILITY STATEMENT
 IS ISSUED

| | |
|--|---|
| <p>I. DEPOSITOR</p> <p>Name: AS ABOVE</p> <p>Address:</p> | <p>II. IDENTIFICATION OF THE MICROORGANISM</p> <p>Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCI MB 41227</p> <p>Date of the deposit or of the transfer¹: 23 June 2003</p> |
| <p>III. VIABILITY STATEMENT</p> <p>The viability of the microorganism identified under II above was tested on 25 June 2004². On that date, the said microorganism was:</p> <p><input checked="checked" type="checkbox"/> ³ viable</p> <p><input type="checkbox"/> ³ no longer viable</p> | |

¹ Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

| | |
|---|---|
| IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED⁴ | |
| | |
| V. INTERNATIONAL DEPOSITARY AUTHORITY | |
| Name: NCIMB Ltd, Address: 23 St Machar Drive Aberdeen AB24 3RY Scotland | Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s):  Date: 28 June 2004 |

⁴ Fill in if the information has been requested and if the results of the test were negative.

CLAIMS

1. A lipolytic enzyme capable of hydrolysing at least a galactolipid and/or capable of transferring an acyl group from a galactolipid to one or more acyl acceptor substrates, wherein the enzyme is obtainable from *Streptomyces* species.
5
2. A lipolytic enzyme capable of hydrolysing polar lipids and/or capable of transferring an acyl group from a polar lipid to one or more acyl acceptor substrates, wherein the enzyme is encoded by a nucleic acid selected from the group consisting of
10 of:
 - a) a nucleic acid comprising a nucleotide sequence shown in SEQ ID No. 3;
 - b) a nucleic acid which is related to the nucleotide sequence of SEQ ID No. 3 by the degeneration of the genetic code; and
 - c) a nucleic acid comprising a nucleotide sequence which has at least 70% identity
15 with the nucleotide sequence shown in SEQ ID No. 3.
3. A lipolytic enzyme according to claim 1 or claim 2 comprising an amino acid sequence as shown in SEQ ID No. 4 or an amino acid sequence which has at least 60% identity thereto.
20
4. A lipolytic enzyme obtainable from the *Streptomyces* strains L130 or L131 deposited under accession numbers NCIMB 41226 and NCIMB 41227, respectively.
5. A lipolytic enzyme according to any one or more of claims 2-4 wherein the enzyme
25 is capable of hydrolysing at least a galactolipid and/or is capable of transferring an acyl group from a galactolipid to one or more acyl acceptor substrates.
6. A lipolytic enzyme according to claim 1 or claim 5 wherein the enzyme is capable of hydrolysing a further polar lipid.
30
7. A lipolytic enzyme according to claim 6 wherein the polar lipid is a phospholipid.

8. A lipolytic enzyme according to claim 1 or claim 5 wherein the lipolytic enzyme is capable of transferring an acyl group from a polar lipid to one or more of the following acyl acceptor substrates: a sterol, a stanol, a carbohydrate, a protein or subunits thereof, or a glycerol.

5

9. A lipolytic enzyme according to any one of the preceding claims wherein the enzyme is a wild-type enzyme.

10. A nucleic acid encoding a lipolytic enzyme comprising an amino acid sequence as shown in SEQ ID No. 4 or an amino acid sequence which has at least 60% identity therewith.

11. A nucleic acid encoding a lipolytic enzyme, which nucleic acid is selected from the group consisting of:

15

- a) a nucleic acid comprising a nucleotide sequence shown in SEQ ID No. 3;
- b) a nucleic acid which is related to the nucleotide sequence of SEQ ID No. 3 by the degeneration of the genetic code; and
- c) a nucleic acid comprising a nucleotide sequence which has at least 70% identity with the nucleotide sequence shown in SEQ ID No. 3.

20

12. Use of a lipolytic enzyme according to any one of claims 1-9 in a process of preparing a lyso-glycolipid, for example digalactosyl monoglyceride (DGMG) or monogalactosyl monoglyceride (MGMG) by treatment of a glycolipid (e.g. digalactosyl diglyceride (DGDG) or monogalactosyl diglyceride (MGDG)) with the lipolytic enzyme according to the present invention to produce the partial hydrolysis product, i.e. the lyso-glycolipid.

25

13. Use of a lipolytic enzyme according to any one of claims 1-9 in a process of preparing a lyso-phospholipid, for example lysolecithin, by treatment of a phospholipid (e.g. lecithin) with the enzyme according to the present invention to produce a partial hydrolysis product, i.e. a lyso-phospholipid.

30

14. Use of a lipolytic enzyme according to any one of claims 1-9 in a process of enzymatic degumming of vegetable or edible oil, comprising treating said edible or vegetable oil with said lipolytic enzyme so as to hydrolyse a major part of the polar lipids.
- 5
15. Use of a lipolytic enzyme according to any one of claims 1-9 in a process of comprising treatment of a phospholipid so as to hydrolyse fatty acyl groups.
16. Use of a lipolytic enzyme according to any one of claims 1-9 in a process of
10 bioconversion of polar lipids to make high value products, wherein said lipolytic is capable of hydrolysing said polar lipids.
17. Use according to claim 16 wherein said high value products are one or more of the following: a carbohydrate ester, a protein ester, a protein subunit ester and a
15 hydroxy acid ester.
18. A method of preparing a foodstuff the method comprising admixing the lipolytic enzyme according to any one of claims 1-9 to one or more ingredients of the foodstuff.
- 20 19. A method according to claim 14 wherein the foodstuff is selected from one or more of the following: eggs, egg-based products, including but not limited to mayonnaise, salad dressings, sauces, ice creams, egg powder, modified egg yolk and products made therefrom; baked goods, including breads, cakes, sweet dough products, laminated doughs, liquid batters, muffins, doughnuts, biscuits, crackers and
25 cookies; confectionery, including chocolate, candies, caramels, halawa, gums, including sugar free and sugar sweetened gums, bubble gum, soft bubble gum, chewing gum and puddings; frozen products including sorbets, preferably frozen dairy products, including ice cream and ice milk; dairy products, including cheese, butter, milk, coffee cream, whipped cream, custard cream, milk drinks and yoghurts;
30 mousses, whipped vegetable creams, meat products, including processed meat products; edible oils and fats, aerated and non-aerated whipped products, oil-in-water emulsions, water-in-oil emulsions, margarine, shortening and spreads including low fat

and very low fat spreads; dressings, mayonnaise, dips, cream based sauces, cream based soups, beverages, spice emulsions and sauces.

20. A method according to claim 19 wherein said foodstuff is a dairy product.

5

21. A method according to claim 19 wherein said foodstuff is an egg or egg-based product.

22. A method according to claim 19 wherein said foodstuff is a dairy product.

10

23. A method of preparing a lysoglycolipid comprising treating a substrate comprising a glycolipid with at least one lipolytic enzyme to produce said lysoglycolipid, wherein said lipolytic enzyme has glycolipase activity and wherein said lipolytic enzyme is obtainable from one of the following genera: *Streptomyces*, *Corynebacterium* and
15 *Thermobifida*.

24. A method of preparing a lysophospholipid comprising treating a substrate comprising a phospholipid with at least one lipolytic enzyme to produce said lysophospholipid, wherein said lipolytic enzyme has phospholipase activity and
20 wherein said lipolytic enzyme is obtainable from one of the following genera: *Streptomyces*, *Corynebacterium* and *Thermobifida*.

25. A method of enzymatic degumming of vegetable or edible oil, comprising treating said edible or vegetable oil with a lipolytic enzyme obtainable from one of the
25 following genera: *Streptomyces*, *Corynebacterium* and *Thermobifida* capable of hydrolysing a major part of the polar lipids.

26. A method of bioconversion of polar lipids to make high value products comprising treating said polar lipids with a lipolytic enzyme obtainable from one of the following
30 genera: *Streptomyces*, *Corynebacterium* and *Thermobifida* to produce said high value products, wherein said lipolytic enzyme is capable of hydrolysing said polar lipids.

27. A method according to claim 26 wherein said high value products are one or more of the following: a carbohydrate ester, a protein ester, a protein subunit ester and a hydroxy acid ester.
- 5 28. A method of preparing a foodstuff comprising admixing at least one lipolytic enzyme with one or more ingredients of a foodstuff wherein said lipolytic enzyme is capable of hydrolysing a glycolipid and/or a phospholipid present in or as at least one of said ingredients and wherein said lipolytic enzyme is obtainable from one of the following genera: *Streptomyces*, *Corynebacterium* and *Thermobifida*.
- 10 29. A method according to any one of claims 23 to 28 wherein said lipolytic enzyme is capable of transferring an acyl group from a glycolipid to one or more acyl acceptor substrates.
- 15 30. A method according to any one of claims 23 to 28 wherein said lipolytic enzyme comprises an amino acid sequence shown as SEQ. ID. No.s 5, 7, 8, 12, 14 or 16 or an amino acid sequence having at least 70% identity therewith or comprises a nucleotide sequence shown as SEQ ID No. 6, 9, 13, 15 or 17 or a nucleotide sequence which has at least 70% identity therewith.
- 20 31. A method according to claim 30 wherein said lipolytic enzyme comprises an amino acid sequence shown as SEQ. ID. No.s 5, 7, or 16 or an amino acid sequence having at least 70% identity therewith.
- 25 32. A method according to claims 30 wherein said lipolytic enzyme comprises an amino acid sequence shown as SEQ. ID. No. 8 or an amino acid sequence having at least 70% identity therewith.
- 30 33. A method according to claims 30 wherein said lipolytic enzyme comprises an amino acid sequence shown as SEQ. ID. No.s 12 or 14 or an amino acid sequence having at least 80% identity therewith.

34. A method according to claim 28 wherein said foodstuff is selected from one or more of the following: eggs, egg-based products, including but not limited to mayonnaise, salad dressings, sauces, ice creams, egg powder, modified egg yolk and products made therefrom; baked goods, including breads, cakes, sweet dough products, laminated doughs, liquid batters, muffins, doughnuts, biscuits, crackers and cookies; confectionery, including chocolate, candies, caramels, halawa, gums, including sugar free and sugar sweetened gums, bubble gum, soft bubble gum, chewing gum and puddings; frozen products including sorbets, preferably frozen dairy products, including ice cream and ice milk; dairy products, including cheese, butter, milk, coffee cream, whipped cream, custard cream, milk drinks and yoghurts; mousses, whipped vegetable creams, meat products, including processed meat products; edible oils and fats, aerated and non-aerated whipped products, oil-in-water emulsions, water-in-oil emulsions, margarine, shortening and spreads including low fat and very low fat spreads; dressings, mayonnaise, dips, cream based sauces, cream based soups, beverages, spice emulsions and sauces.

35. A method according to claim 34 wherein said foodstuff is a baked product and at least one of said ingredients is a dough.

36. A method according to claim 34 wherein said foodstuff is an egg or egg-based product.

37. A method according to claim 34 wherein said foodstuff is a dairy product.

38. Use of a lipolytic enzyme in a substrate for preparing a lysoglycolipid wherein said lipolytic enzyme has glycolipase activity and wherein said lipolytic enzyme is obtainable from one of the following: *Streptomyces*, *Corynebacterium* and *Thermobifida*.

39. Use of a lipolytic enzyme in a substrate for preparing a a lysophospholipid wherein said lipolytic enzyme has phospholipase activity and wherein said lipolytic enzyme is

obtainable from one of the following: *Streptomyces*, *Corynebacterium* and *Thermobifida*.

40. Use of a lipolytic enzyme obtainable from one of the following genera:
5 *Streptomyces*, *Corynebacterium* and *Thermobifida* for enzymatic degumming of vegetable or edible oil so as to hydrolyse a major part of the polar lipids.

41. Use of a lipolytic enzyme obtainable from one of the following genera:
10 *Streptomyces*, *Corynebacterium* and *Thermobifida* in a process comprising treatment of a phospholipid so as to hydrolyse fatty acyl groups.

42. Use of a lipolytic enzyme in the bioconversion of polar lipids to make high value products, wherein said lipolytic enzyme is capable of hydrolysing said polar lipids and wherein said lipolytic enzymes is obtainable from one of the following genera:
15 *Streptomyces*, *Corynebacterium* and *Thermobifida*.

43. Use according to claim 42 wherein said high value products are one or more of the following: a carbohydrate ester, a protein ester, a protein subunit ester and a hydroxy acid ester.
20

44. Use of a lipolytic enzyme obtainable from one of the following genera:
25 *Streptomyces*, *Corynebacterium* and *Thermobifida* in the preparation of a foodstuff, wherein said lipolytic enzyme is capable of hydrolysing a glycolipid and/or a phospholipid.

45. Use according to claim 38 to 44 wherein said lipolytic enzyme is capable of transferring an acyl group from a glycolipid to one or more acyl acceptor substrates.

46. Use according to any one of claims 38 to 44 wherein said lipolytic enzyme
30 comprises an amino acid sequence as shown in any one of SEQ. ID. No.s 5, 7, 8, 12, 14 or 16 or an amino acid sequence having at least 70% identity therewith or

comprises a nucleotide sequence shown as SEQ ID No. 6, 9, 13, 15 or 17 or a nucleotide sequence which has at least 70% identity therewith.

5 47. Use according to claim 46 wherein said lipolytic enzyme comprises an amino acid sequence as shown in any one of SEQ. ID. No.s 5, 7, or 16 or an amino acid sequence having at least 70% identity therewith.

10 48. Use according to claims 46 wherein said lipolytic enzyme comprises an amino acid sequence shown as SEQ. ID. No. 8 or an amino acid sequence having at least 70% identity therewith.

15 49. Use according to claims 46 wherein said lipolytic enzyme comprises an amino acid sequence shown as SEQ. ID. No.s 12 or 14 or an amino acid sequence having at least 80% identity therewith.

50. Use according to any one of claim 38 wherein said lysoglycolipid is DGMG or MGMG.

20 51. Use according to claim 44 wherein said foodstuff is a dairy product

52. Use according to claim 51 wherein said foodstuff is an egg or an egg-based product and wherein said lipolytic enzyme is capable of transferring an acyl group to one or more acyl acceptor substrates to reduce the one or more of the following detrimental effects: off-odours and/or off-flavours and/or soapy tastes.

25 53. Use according to claim 51 wherein said foodstuff is a baked product

54. Use according to claim 38 wherein said substrate is an edible oil.

30 55. A lipolytic enzyme as hereinbefore described with reference to the accompanying description and figures.

56. A method as hereinbefore described with reference to the accompanying description and the figures.

57. A use as hereinbefore described with reference to the accompanying description
5 and figures.

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FIGURE 1

SEQ ID No. 1:

5

GACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCC
GCCTGGGGAGTACGGCCGCAAGGCTAAAACCTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGCG
GAGCATGTGGCTTAATTCGACGCAACCGGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGG
10 CCAGAGATGGTCGCCCCCTTGTGGTGGTGTACAGGTGGTGCATGGCTGTCGTGACCTCGTGTGCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCTTATCCTGTGTTGCCAGCGGATCCCTTCG
GGGGTGCCGGGACTCACGGGAGACTGCCGGGTCAACTCGGA

FIGURE 2

15

SEQ ID NO. 2:

GACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCC
20 GCCTGGGGAGTACGGCCGCAAGGCTAAAACCTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGCG
GAGCATGTGGCTTAATTCGACGCAACCGGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGG
CCAGAGATGGTCGCCCCCTTGTGGTGGTGTACAGGTGGTGCATGGCTGTCGTGACCTCGTGTGCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCTTATCCTGTGTTGCCAGCGGATCCCTTCG
GGGGTGCCGGGACTCACGGGAGACTGCCGGGTCAACTCGGA

25

FIGURE 3

SEQ ID NO. 3:

5 ACAGGCCGATGCACGGAACCGTACCTTTCCGCAGTGAAGCGCTCTCCCCCATCGTTCCG
 CGGGACTTCATCCGCGATTTTGGCATGAACACTTCTTCAACGCGCGTAGCTTGCTACAA
 GTGCGGCAGCAGACCCGCTCGTTGGAGGCTCAGTGAGATTGACCCGATCCCTGTCGGCCG
 CATCCGTCATCGTCTTCGCCCTGCTGCTCGCGCTGCTGGGCATCAGCCCGGCCAGGCAG
 CCGGCCCGGCTATGTGGCCCTGGGGGATTCTATTCTCGGGCAACGGCGCCGGAAGTT
 10 ACATCGATTGAGCGGTGACTGTACCGCAGCAACAACCGGTACCCCGCCCGCTGGGCGG
 CGGCCAACGCACCGTCTCTTACCTTCGCGGCTGCTCGGGAGCGGTGACCACGGATG
 TGATCAACAATCAGCTGGGCGCCCTCAACCGGTCCACCGGCTGGTGAGCATCACCATCG
 GCGGCAATGACGCGGGCTTCGCGGACCGGATGACCACCTGCGTACCAGCTCGGACAGCA
 CCTGCCTCAACCGGCTGCCCACCGCCACCAACTACATCAACACCACCTGCTCGCCGGC
 15 TCGACGCGGTCTACAGCCAGATCAAGGCCCGTGCCCCAACGCCCGGTGGTCTCCTCG
 GTACCCGCGCATGTACCTGGCCTCGAACCCCTGGTACTGCCTGGGCTGAGCAACACCA
 AGCGCGCGGCATCAACACCACCGCCGACCCCTCAACTCGGTGATCTCCTCCCGGGCCA
 CCGCCACGGATTCCGATTGCGCGATGTCCGCCCGACCTTCAACAACCACGAAGTGTCT
 TCGGCAACGACTGGCTGCACTCACTCACCTGCCGGTGTGGGAGTCGTACCACCCACCA
 20 GCACGGGCCATCAGAGCGGCTATCTGCCGTCCTCAACGCCAACAGCTCGACCTGATCAA
 CGCACGGCCGTGCCCGCCCGCGCGTACGCTCGGCGCGGGCGCCGAGCGCGTTGATCA
 GCCACAGTGCCGGTGACGGTCCACCCTCACGGTCGAGGGTGTACGTCACGGTGGCGCC
 GCTCCAGAAGTGGAACTCAGCAGGACCGTGGAGCCGTCCTGACCTCGTGAAGAAGT
 CGGGGTGACGCTGATCACCCCTCCCCGTAGCCGGGGGCGAAGGCGCGCCGAAGTCTT
 25 GTAGGACGTCCAGTCGTGCGGCCCGCGTGGCCACCCTCCGCGTAGACCGCTTCCATGCT
 CGCCAGCCGGTCCCCGCGGAAGTCCGGTGGGGATGTCCGTGCCCAAGGTGGTCCCGGTGGT
 GTCCGAGAGCACCGGGGGCTCGTACCGGATGATGTGCAGATCCAAAGAATT

FIGURE 4

30

SEQ ID NO. 4:

MRLTRLSAASVIVFALLALLGISPAQAAGPAYVALGDSYSSGNGAGSYIDSSGDCHRSN
 NAYPARWAAANAPSSFTFAACSGAVTTDVINNLGALNASTGLVSITIGGNDAGFADAMTT
 35 CVTSSDSTCLNRLATATNYINTLLARLDAVYSQIKARAPNARVVVLGYPRMYLASNPWYC
 LGLSNTKRAAJNTTADTLNSVISSRATAHGFRFGDVRPTFNNHELFFGNDWLHSLTLPWE
 SYHPTSTGHQSGYLPVLNANSST

FIGURE 5

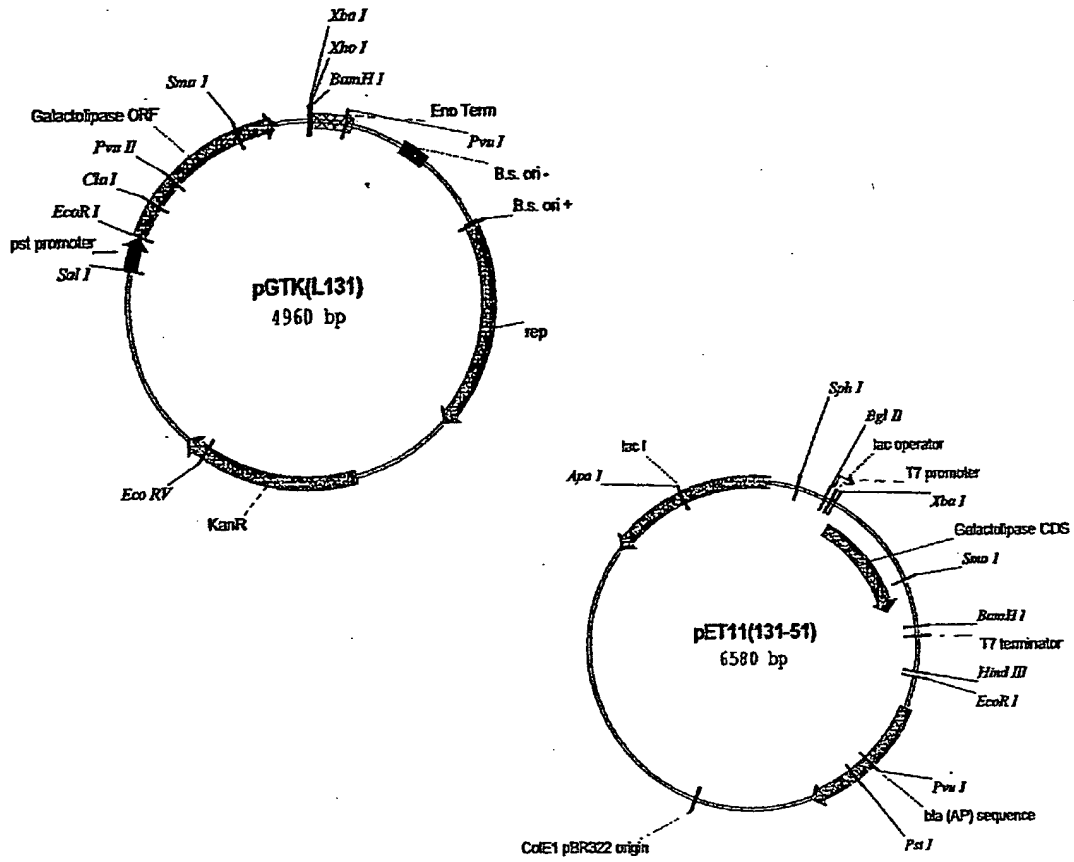


FIGURE 6

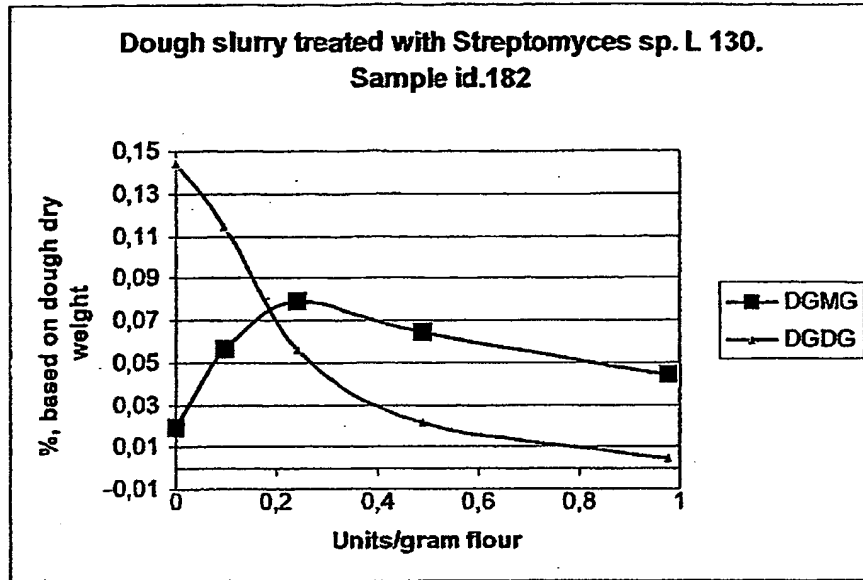


FIGURE 7

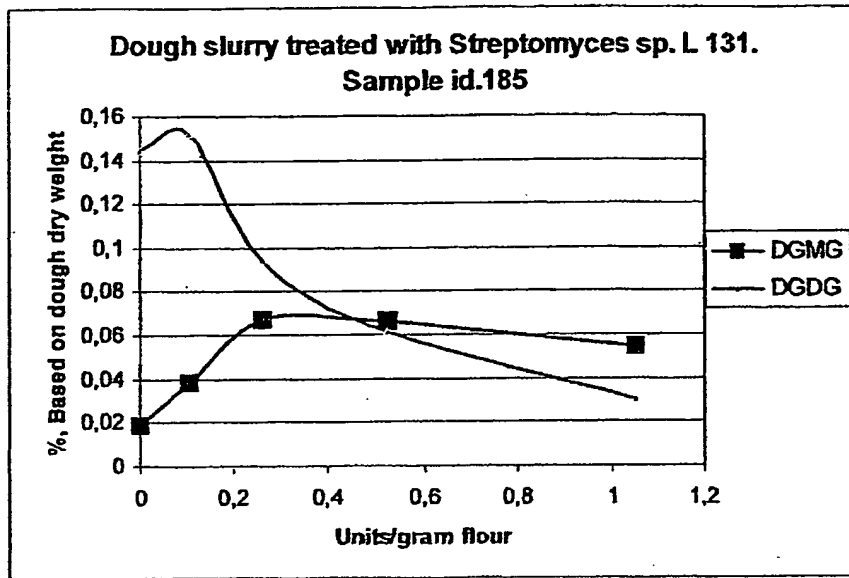


FIGURE 8

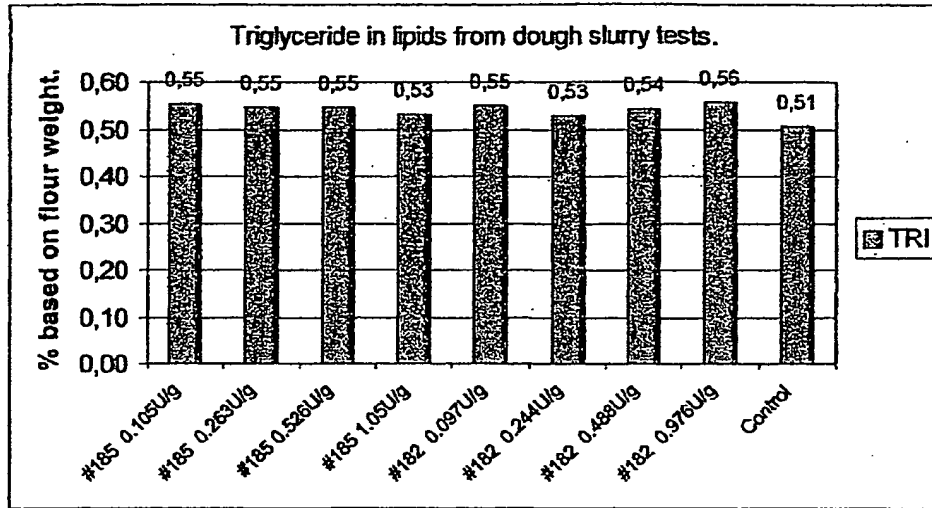


FIGURE 9

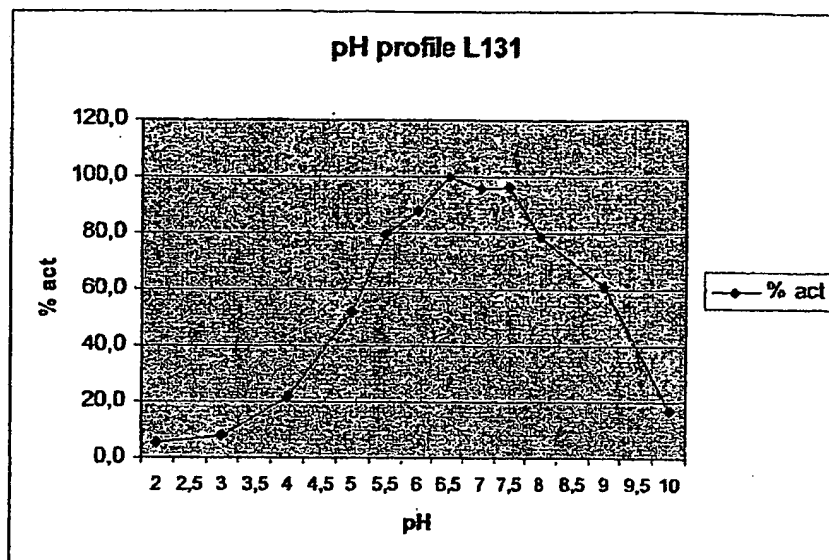
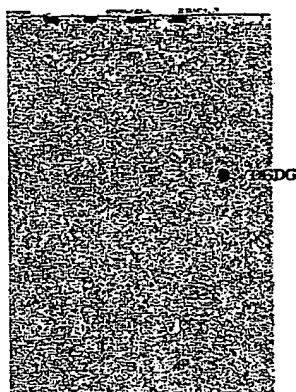
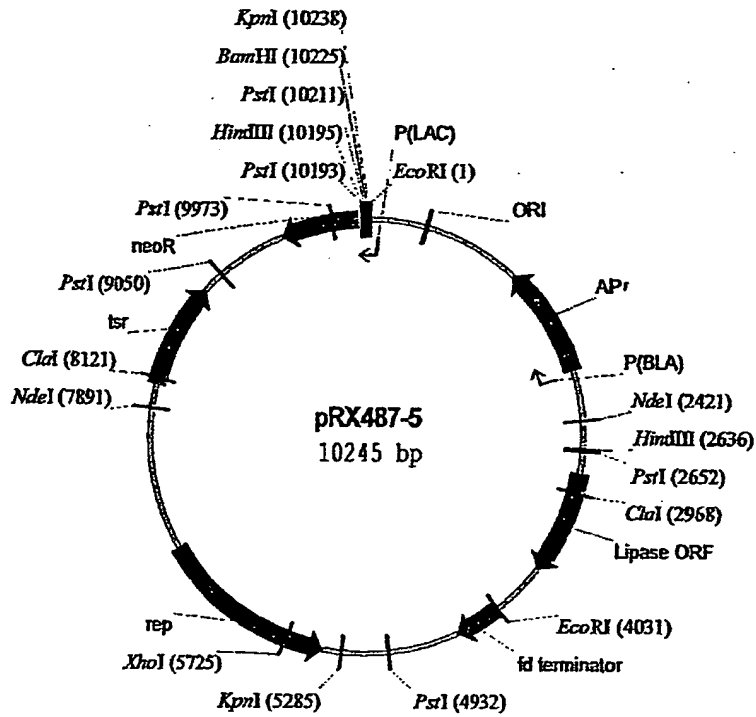


FIGURE 10



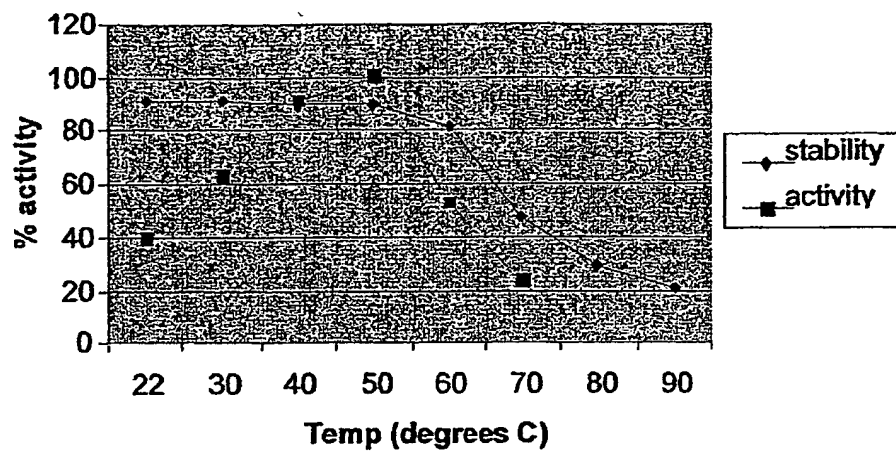
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Figure 11



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FIGURE 12



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FIGURE 13

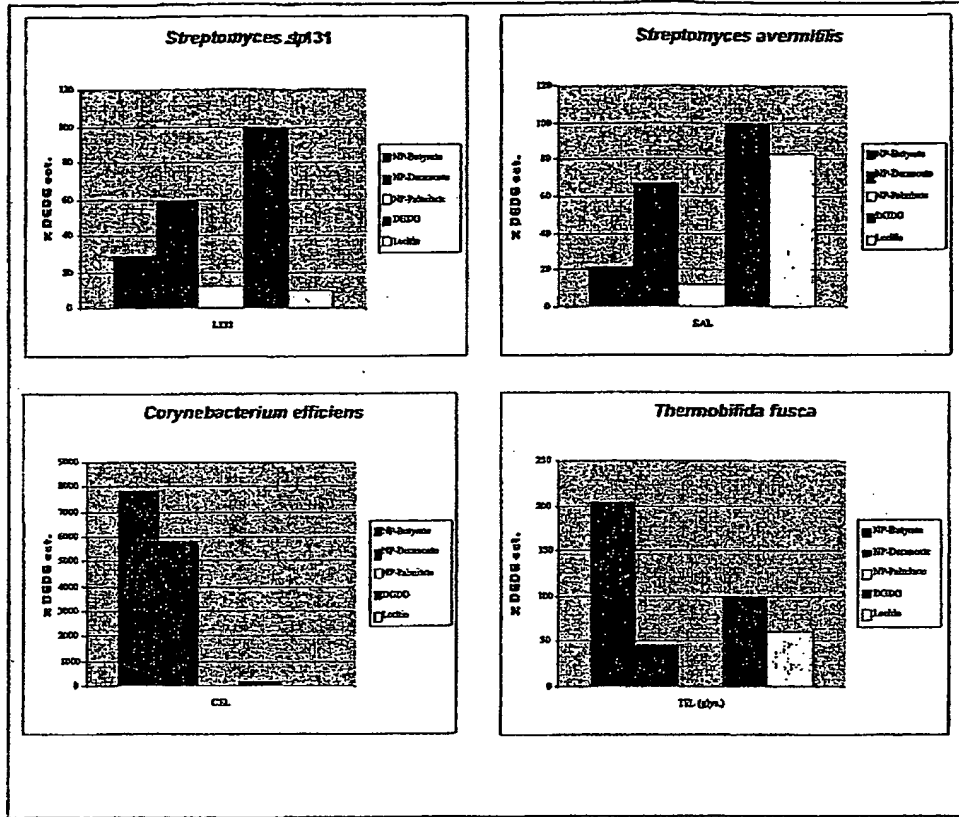


FIGURE 14

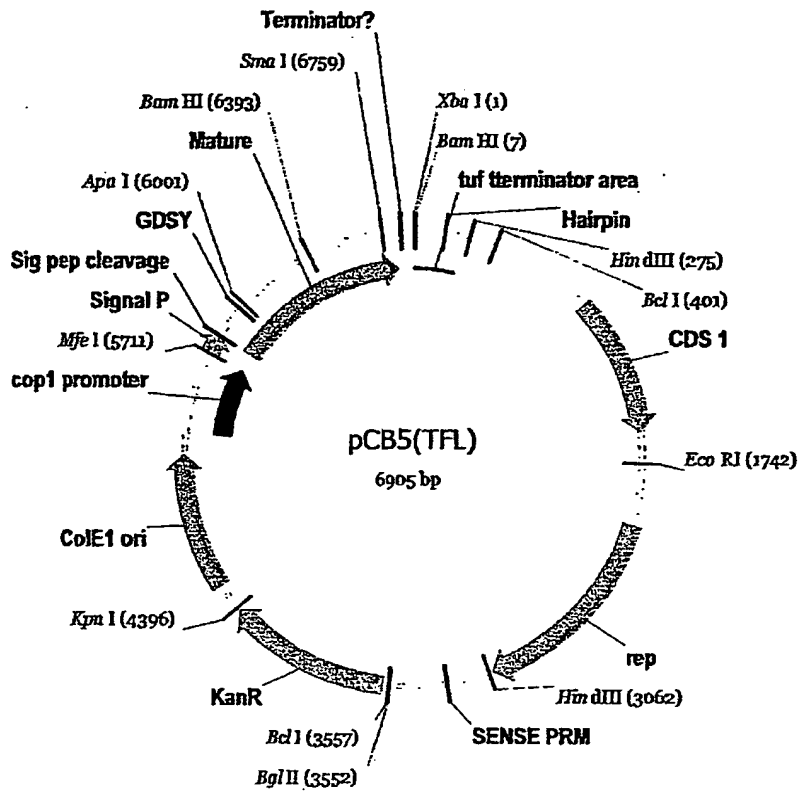


FIGURE 15

- 1. L131
- 2. *S. avermitilis*
- 3. *T. fusca*
- 5 4. Consensus

```

1
10 1 (1) -----MRLTRSLSAASVIVFALLLALLGISPAQAAG-----
2 (1) -----MRRSRITAYVTSLLLAVGCALTGAATAQASPA-----
3 (1) VSGSPRAATRRLFLGIPALVLTALTLLVAVPTGRETLWRMWCEATQDW
4 (1) MRRSRFLA ALILLFLA AL GAA ARAAP

15 51
1 (32) -----P-AYVALGDSYSSGNGAGSYID
2 (33) -----AAATGYVALGDSYSSGVGAGSYLS
3 (51) CLGVPVDSRGQPAEDGEFLLLSPVQAATWGNYYALGDSYSSGDGARDYYP
4 (51) A A YVALGDSYSSG GAGSY

20 101
1 (53) SSGD---CHRSNNAYPARWAAANAP---SSFTFAACSGAVTTDVIN----
2 (57) SSGD---CKRSSKAYPYLWQAAHSP---SSFSFMACSGARTGDVLA----
3 (101) GTAVKGGCWRSANAYPELVAEAYDFA---GHSFLACSGQRGYAMLDAIDE
4 (101) SSGD C RSTKAYPALWAAAHA SSFSF ACSGARTYDVIA

25 151
1 (93) --NQLGALNAST--GLVSITIGENDAGFADAMTTCVTS-----SDSTCL
2 (97) --NQLGTLNSST--GLVSLTIGENDAGFSDVMTTCVLQ-----SDSACL
3 (149) VGSQLDWNSPHT--SLVTIGIGNDLGFSTVLKTCMVR-----VPLLDS
30 4 (151) QL LNS T LVSITIGENDAGFAD MTTCVL SDSACL

35 201
1 (133) NRLATATNYINTLLA-----RLDAVYSQIKARAPNARVVVLGYPRMY
2 (137) SRINTAKAYVDSTLPG-----QLDSVYTAISTKAPSAHVAVLGYPRFY
3 (191) KACTDQEDAIRKRMKF---ETTFEELISEVRTRAPDARILVVGYPRI
4 (201) RIA AK YI TLPA RLDSVYSI TRAP ARVVVLGYPRIY

40 251
1 (176) LASNPWYCLGLSNTKRAINTTADTLNSVISSRATAH-----GF
2 (180) KLGGSCLAGLSETKRSAINDAADYLSAIKRAADH-----GF
3 (237) PEEPTGAYYTLASNQRWLNETIQEFNQQLAEAVAVHDEEIAASGGVGSV
4 (251) SG LGLS TKRAAINDAAD LNSVIAKRAADH GF

45 301
1 (215) RFGDVRPTFNNHELFGNDWLHSLTLP-----VWESYH
2 (218) TFGDVKSTFTGHEICSSSTWLHSLDLLN-----IGQSYH
3 (287) EFVDVYHALDGHEIGSDEPWVNGVQLRDLATG-----VTVDRSTFH
4 (301) TFGDV TF GHELCSA PWLHSLTLP V SYH

50 351
1 (248) PTSTGHQSGYLPVLNANSST-----
2 (252) PTAAGQSGGYLPVMNSVA-----
3 (328) PNAAGHRAVGERVIEQIETGPGRPLYATFAVVAGATVDTLAGEVG
4 (351) PTA GHAAGYLPVLSI T

```

FIGURE 16

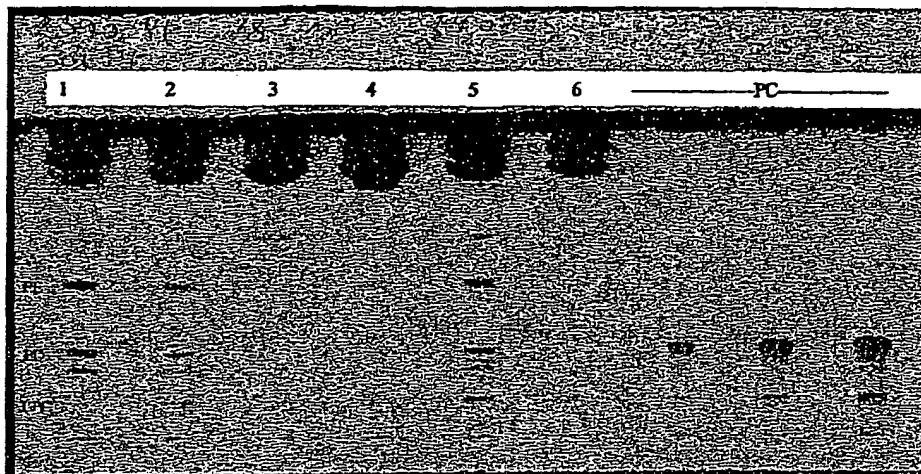
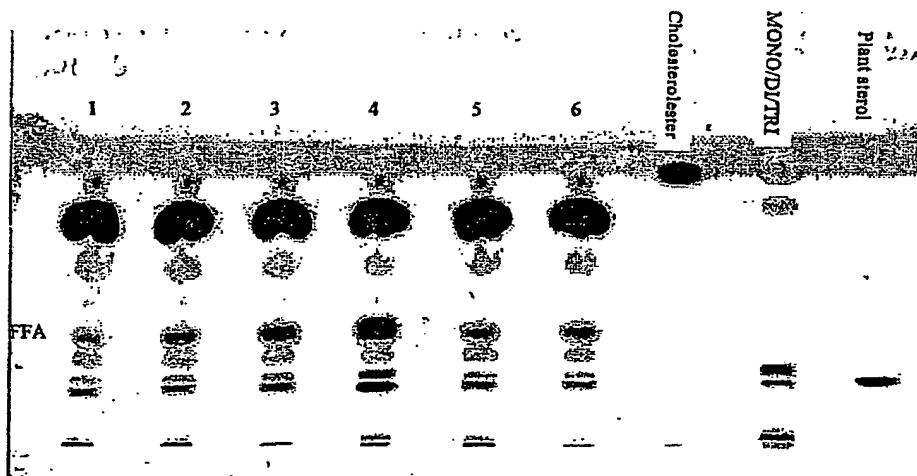


FIGURE 17



PROTEIN

FIELD OF INVENTION

The present invention relates to a novel lipolytic enzyme, in particular a novel lipolytic
5 enzyme, and nucleotide sequences encoding same. The present invention also relates
to methods of production of the novel lipolytic enzyme and to uses thereof. The
present invention also relates to methods and uses of a lipolytic enzyme.

TECHNICAL BACKGROUND

10

The beneficial use of lipolytic enzymes active on glycolipids in bread making was
taught in EP 1 193 314. It was taught that the partial hydrolysis products the lyso-
glycolipids were found to have very high emulsifier functionality. However, the
enzymes taught in EP 1 193 314 were also found to have significant non-selective
15 activity on triglycerides which resulted in unnecessarily high free fatty acid.

20

A lipolytic enzyme from *Fusarium oxysporum* having phospholipase activity has been
taught in EP 0 869 167. This lipolytic enzyme has high triacylglyceride hydrolysing
(lipase) activity. This enzyme is now sold by Novozymes A/S (Denmark) as Lipopan
F™.

25

WO02/00852 discloses five lipase enzymes and their encoding polynucleotides,
isolated from *Fusarium venenatum*, *F. sulphureum*, *Aspergillus berkeleyanum*, *F.*
culmorum and *F. solani*. All five enzymes are described as having triacylglycerol
hydrolysing activity, phospholipase and galactolipase activity.

30

Lipolytic enzyme variants, with specific amino acid substitutions and fusions, have
been produced; some of which have an enhanced activity on the polar lipids compared
to the wildtype parent enzymes. WO01/39602 describes such a variant, referred to as
SP979, which is a fusion of the *Thermomyces lanuginosus* lipase, and the *Fusarium*
oxysporum lipase described in EP 0 869 167. This variant has been found to have a

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